METABOLIC HORMONE LEVELS AND IMMUNOCOMPETENCE OF NEONATAL HARBOR SEALS (PHOCA VITULINA) IN REHABILITATION SETTINGS COMPARED TO WILD HARBOR SEAL PUPS

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A

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ABSTRACT

Health of harbor seal pups in rehabilitation and in the wild were compared using two metabolic hormones (cortisol and total thyroxine, TT4), two cellular immunity components (lymphocytes and eosinophils) and morphometric measurements. Neonatal harbor seals in two rehabilitation facilities were compared to wild harbor seal pups. Permanently captive harbor seals housed at the Alaska SeaLife Center were also studied.

High levels of cortisol at weaning suggest changes in the stress response may be due to diet adjustments in pups during rehabilitation. The lower cortisol concentrations post-weaning suggest that pups in rehabilitation had overcome the challenge of pre-weaning diet, handling or environment and avoided chronic stress. TT4 concentrations were higher in wild pups, likely attributed to a more energetically demanding life in a dynamic environment.

The rehabilitated pups showed lower lymphocyte counts and higher eosinophil counts compared to wild pups. Wild harbor seal pups were heavier and longer than post-weaned pups in rehabilitation. Animals in rehabilitation are possibly compromised at stranding, but it is also possible that current rehabilitation practices do not mimic what a healthy pup would receive from maternal investment, thus pups undergoing rehabilitation likely remain smaller and possibly immunologically compromised despite repeated and constant care in rehabilitation.

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Chapter 1 Introduction to Harbor Seal Biology, Endocrinology, Immunology, Physiology and Husbandry

Harbor seals (Phoca vitulina) are one of the most geographically widespread species of all the phocids. In the Pacific, they range from southern California to the Alaskan arctic, crossing the Pacific to the eastern Russian peninsula and south to the Japanese islands. Atlantic harbor seals range from the Canadian Arctic south into the New England states and over into the Svalbard peninsula, Greenland and around Great Britain north to Norway and Iceland (Riedman, 1990; Wynne and Schwartz, 1999). Harbor seals were one of the resources impacted by the 1989 Exxon Valdez oil spill (EVOS) in Prince William Sound, Alaska. Even 14 years later, the species is labeled as "not recovering" by the Alaska Department of Fish and Game, although it is recognized that the decline actually began before the 1989 oil spill (Rice et al. 1993; Loughlon, 1995).

Pups are usually born in the early spring to early summer but births have been recorded from March to September. Mating time is variable, ranging from June to October, depending upon subspecies, and gestation is 9 to 10 months (Dierauf and Dougherty, 1983; Wynne, 1997). Birthing substrate can vary from rocky shores to glacial ice to sand beaches. Average pups at birth weigh between 10 to 12 kgs (Dierauf et al., 1986; Katona et al., 1993; Wynne, 1997). Adult weights range from 110 to 140 kgs. Some subspecies are born with lanugo, or natal coat, but most shed their lanugo in utero (Wynne, 1997). Phoca vitulina richardsii, the harbor seal subspecies on the west coast of the United States ranging from California to Alaska, is commonly born with its lanugo

(Dierauf et al., 1983; Dierauf and Dougherty, 1983; Gage, 1994). Harbor seal pups are precocious at birth (Dierauf et al., 1986; Riedman, 1990). They can enter the sea almost immediately after birth (Muelbert and Bowen, 1993). As precocial as harbor seals pups are at birth, if they become separated from their mothers at too early an age, they are susceptible to malnutrition, disease, or predation (Roletto, 1993).

Analysis of body condition via morphometrics can give a gross indication of an animal's body condition. Morphometrics are measurements of the body of the animal. Weight, standard length, and axillary and pelvic girth are common morphometrics measured from seals. Average lengths of harbor seal pups range from 60 to 75 cm (Katona et al., 1993; Wynne, 1997). Harbor seal pups will sometimes be admitted to rehabilitation centers underdeveloped in terms of normal documented birth weight. However, below normal documented weights do not necessarily mean they are premature. There have been near full term pups delivered by cesarean section admitted to rehab centers from Alaska Native subsistence takes that weighed over 9 kgs (ASLC rehab data, 2000). Girths will vary greatly dependent on age and overall body condition of the pup. Representatives from differing subspecies and different geographical areas will fluctuate within documented morphometric ranges.

Neonates are defined as newborn animals, under 28 days of age and wean anywhere from 4 to 6 weeks after birth (Roletto, 1993). The nursing period is important for two primary reasons; acquisition of immunity and fattening. Young animals receive the majority of their immunity through maternal investment, in this case, milk. If an animal is denied that initial immunity, the possibility of survival decreases exponentially

(Tizard and Shubot, 2000). Proper fat and protein deposition is crucial for pup survival (Dierauf, et al., 1983). Fattening is a critical factor in the life of a phocid neonate (Iverson, et al., 1995). Harbor seal milk is approximately 45% fat (Riedman, et al., 1990). This fat percentage helps in giving the pup enough energy storage to not only thermoregulate, but also to later survive the post-weaning fast. Composition of the pup's blubber changes during the lactation period in that percent fat increases while water and protein decrease (Bowen et al., 1992). This may help to provide sufficient reserves of energy during the post-weaning fast (Oftedal, et al., 1995; Ortiz, et al. 1978; Bowen, 1991), while providing water ingestion early in lactation so that the pup does not need to rely on deriving water metabolically, thus diminishing energy reserves. Neonates also have problems with thermoregulation. High amounts of energy are expended to try to stay thermal-neutral post-utero. Due to low amounts of body fat, especially if no nursing has occurred, the pup can chill and die of hypothermia. Even though pups are born without blubber layers, they do possess stores of brown fat, or fatty, adipose tissue that aids in maintaining body heat and fuel reserves (Riedman, 1990).

Harbor Seal Husbandry

Many marine mammal rehabilitation centers admit neonate harbor seals. The most common reason for harbor seal admission to centers is illegal beach pick-ups where the public is unaware that there may be the mother hunting offshore and see only a mewing pup on the beach, seemingly alone. Once a pup is harassed by humans or dogs on a beach, the mother will surrender the pup and leave the area. Rehabilitation centers attempt to educate the public to leave a pup alone and watch it for a 24 hour period,

unless there is a possible fatal disturbance such as birds of prey or large dogs, or external injury to the pup (ASLC, 2001; TMMC, 1994). If mother does not return to nurse the pup, the pup is then deemed abandoned.

Neonates brought into rehabilitation centers are administered electrolytes and then a high fat fabricated milk matrix formula after stabilization. The powdered, commercially produced milk matrix is blended with salmon oil. The salmon oil acts as the high fat compound. Fabricating a milk matrix with the correct nutrient and fat content has been a focus of many marine mammal rehabilitation centers for years. Depending upon health status, positive weight gain and stage of teeth eruption, pups are weaned from the milk formula to solid fish (Townsend and Gage, 2001). This weaning process varies widely and is determined by facility and animal behavior. Certain pups take longer to wean than others. Some rehabilitation facilities use slower weaning processes to help ensure uninterrupted nutrients and weight gain to the weanling simulating the natural proposed duration of lactation, approximately 4-6 weeks. In this method fish are introduced to the animal while formula is still being administered on a regular basis. The pup may still be receiving formula via stomach gavage while it is also force-fed fish. At a certain weight, the facility decides to begin decreasing formula and introducing fish to the diet. A period of time may lapse when the pup is fed both formula and fish prior to complete weaning when the animal is either force-fed fish or foraging independently. Other facilities choose abrupt weaning, to mimic what mother harbor seals would do in the wild to the pup. If a set of caveats are established such as proper body weight, defecation consistency, blood chemistries within documented ranges, energy/activity level and overall good health

status, all formula is aborted and the only diet for the pup is fish. Most facilities use a method that is a combination of both strategies

Marine mammal rehabilitation facilities take in stranded, injured or harassed marine mammals of all ages. Neonates are the most delicate of all rehab patients due to lack of maternal investment, poor body condition and immature immune system. Age, health state, feeding mode and stranding or injuries all differ amongst pups admitted to rehabilitation facilities. This makes preliminary assessment and stabilization of the pup time-consuming. Upon admittance, pups are examined physically, weighed, standard length, girth and axillary measurements are taken and presence of lanugo and umbilicus are recorded (Townsend and Gage, 2001). Subsequent diagnosis and feeding implementation is decided upon after initial observations and overall body condition.

Harbor Seal Endocrinology

A useful documentation of metabolic hormones investigated to date for many marine mammals, including animals admitted into rehabilitation facilities, is located in the CRC Handbook of Marine Mammal Medicine (Haulena et. al.,1998). Immunocompetence in an individual animal or species is a complex series of metabolic and physiological interactions. It is dependent on environmental, nutritional and endocrinological factors. Hormones, such as cortisol (an adrenal hormone) and thyroxine, measured as total T4 (TT4, a thyroid hormone), are good indicators of metabolic state and potential stressors to an animal's physiological system (Oki and Atkinson, 2004). Spikes or depressions of these metabolic hormones can aid in determining the well-being of an animal's system. Hormones such as cortisol and TT4, if levels are altered due to

stress, can cause changes in metabolic rate, calcium absorption and blood pressure control (Bernal and Refetoff, 1977; McDonald and Capen, 1989; Haulena et al., 1998; Woldstad and Jenessen, 1998; Bondy and Cohn, 2002). Cortisol and TT4 abnormalities, such as hyperthyroidism or euthyroidism, over time have been found to cause forms of immunosuppression in pinniped species (Bernal and Refetoff, 1977; Haulena et al., 1998; Bondy and Cohn, 2002).

Cortisol

Cortisol is the dominant circulating glucocorticoid that maintains a level of homeostasis in the body to aid against environmental pressures in all marine mammals (St. Aubin et al., 1996; Ortiz and Worthy, 2000). Cortisol is a glucocorticoid steroid that is released directly from the adrenal cortex when stimulated by the anterior pituitary gland. The adrenal cortex is well developed in neonatal harbor seals, which may explain precociousness in the species (St. Aubin, 2001). Glucocorticoids promote the action of gluconeogenesis, the metabolic conversion of amino acids into glucose, which ultimately leads to increasing blood glucose levels (Oki, 2001). Most metabolic glucocorticoids have a catabolic rather than an anabolic effect (Bondy and Cohn, 2002). Chronic exposure to cortisol can inhibit the inflammatory response and reduce the immune response efficiency in an organism. Cortisol acts upon protein, lipid and carbohydrate mobilization and metabolism, aids in varied adaptations of the immune reaction to a number of distresses, and limits cell and tissue damage by doing so (Eckert et al.,1988). Glucocorticoids have been well documented for their role in the stress response (Selye, 1946; St. Aubin and Dierauf, 2001). Cortisol is noted as an indicator of health status in an

animal due to levels rising during times of stress (Oki, 2001). Cortisol usually increases within 25 to 30 minutes after initial stressor (Atkinson, unpublished data, 2002; St. Aubin, 2001). Rising cortisol levels affect immune system functions shown as classic stress leukograms such as eosinopenia, circulating mature neutrophils and lymphocytopenia (St. Aubin, 2001; Bondy and Cohn, 2002). Cortisol has also been discussed as having a marked difference in circulating concentrations based on circadian rhythms (Oki and Atkinson, 2004). In a study by Gardiner and Hall (1997), there were no differences in cortisol levels in captive harbor seals when age, gender and season were compared. However, in the same study, harbor seal cortisol showed increases at night and decreases prior to dawn with the lowest levels in early afternoon. Cortisol levels vary with time of day and cortisol responses correlate with baseline levels, although the percentage increase declined during rehabilitation (Gulland et al., 1999). The study later goes on to state that increasing baseline cortisol could be due to a failure to adapt to captivity resulting in chronic stress. In the Gulland study, this is in reference to animals that died in the rehabilitation setting, referring to chronic stress.

Thyroxine

Thyroxine (T4) is one of two thyroid hormones secreted by the thyroid glands, stimulated by thyroid stimulating hormone (TSH). Thyroxine influences aspects of reproduction, growth, metabolism and development of the immune system (Hall et al., 1998; Woldstad and Jenessen, 1999; St. Aubin and Geraci, 1987). Many of these actions occur cooperatively with other hormones; the thyroid hormones enhance their effectiveness. Thyroxine is converted to triiodothyronine (T3), which is more readily

taken up by various organs of the body and has higher biological activity than T4 in many mammalian species (Thomson and McGirr, 1976; St. Aubin and Geraci, 1987; Woldstad and Jenessen, 1999). Many factors such as diet influence the thyroid state in mammals, which in turn may influence processes controlled by other hormones (Norris, 1997). During periods of distress, thyroid hormone concentrations will decrease as a part of a larger feedback system to preserve energy reserves (St. Aubin and Geraci, 1987). Changes in circulating glucocorticoids can influence thyroid hormone secretion and the conversion of T4 into T3, the more physiologically active form (Re et al., 1976). Results from studies of beluga whales brought into captivity by St. Aubin and Geraci (1987) suggest that the glucocorticoid component of the stress response realigns to conserve thyroid hormone balance, or the glucocorticoid response to stress reduces circulating thyroid hormones. In the same study, TT4 levels did not change for almost 20 hours post-capture, possibly showing that the stress response is more conservative in marine mammals in terms of glucocorticoids and TT4 when compared to terrestrial mammals.

Glucocorticoids decrease circulating levels of thyroid hormones by inhibiting the monodeiodination of T4 (Re et al., 1976). Due to the negative feedback loop present in hormone relationships, decreases in circulating glucocorticoid hormone levels elicit a compensatory increase in thyroid concentrations (St. Aubin and Geraci, 1987). Most measurements of thyroid hormones, such as T4, in all ages of an animal are remarkably constant post weaning (Woldstad and Jenessen, 1999). However, marine mammal neonates may differ. Amoroso et al. (1965) found that common and grey seal neonates exhibit variable to large neonatal thyroid gland size, which decreases shortly

after birth. Engelhardt and Ferguson (1979) found that newborn harp seals had the highest T4 levels compared to other species of seals, followed by a rapid decline, almost a 40% decrease at 3 weeks of age. This seems to indicate a calorigenic function to mobilize brown fat and glycogen stores for thermoregulatory processes (Blix et al., 1979, Leatherland and Ronald, 1979). Leatherland and Ronald (1979) state that harp seal neonates have the highest T4 levels in age 1 day to 5 days, compared to other pups and to adults. Woldstad and Jenessen (1999) show the same results in grey seal pups, that plasma T4 levels are highest in pups aged 2 days and then dropping to a stable level thereafter. The same study predicts the decreasing of T4 after 3 days is due to the reduction of thyroid secretory action. In a study by Harrison et al. (1962) in newborn harbor seals there is a noted histology of active thyroid hormone activity. Hall et al. (1998) concluded that grey seal pup thyroxine levels were higher in pre-weaned pups in comparison to adults but not higher to post-weaned pups. Pups reached adult levels of thyroxine at approximately 18 days of age. There is a possibility that female sex hormones, such as progesterone, may be stimulating thyroid activity in fetal or newborn pups (Hall et al., 1998). An exception is seen in neonates where there is a recorded period of thyrotoxicosis. Thyrotoxicosis is a condition resulting form an excess of circulating thyroid hormones (T4 and/or T3) (Walker, 1995). Thyrotoxicosis is caused by a transient rise in TSH (thyroid stimulating hormone) in the first few days of life (Thomson and McGirr, 1976). Congenital thyrotoxicosis can last up to three months in human neonates and is due to transplacental passage of the stimulating maternal antibodies of the IgG class, which can cause infant mortality if untreated (Smith, 2000).

The variety of thyroxine information in neonatal seals warrants further research to build upon the information already known, and to continue understanding hormone activity and immune function in young phocine species.

Stress and Hormones

The body recognizes stress in different ways and the endocrinological system reacts accordingly. Thyroid secretion diminishes and glucocorticoid secretions increase (St. Aubin and Geraci, 1988). Glucocorticoids such as cortisol have three basic functions in relation to stress; to alter carbohydrate metabolism and increase circulating substances for energy, to allow catecholamines to act on metabolic pathways and blood vasculature, and to provide protective adaptations to distress by limiting immunological reactions, including inflammation to minimize cell and tissue damage (St. Aubin and Dierauf, 2001). The primary endocrine components are ACTH (corticotropin), epinephrine and catecholamines. The secondary endocrine components of the stress response include the glucocorticoids (cortisol and corticosterone). The tertiary endocrine components respond in a cascading fashion to the primary endocrine components and among them, thyroxine (Dunn, 1995; Lovallo, 1997; Breazile, 1988). The most complicated aspect of studying a stress response in an animal is the ability to determine the difference between a true stress state versus an unstressed, or baseline state. Many diagnostic techniques (capture, handling, sampling, blood draws) can bring about a stress state in an animal, but not necessarily in every animal tested. With efficient capturing techniques and sampling done within 10 minutes of capture, close to baseline values of hormones and other circulating molecules can still be obtained in most mammals (St. Aubin and Dierauf, 2001).

Thomson and Geraci (1986) measured cortisol in bottlenose dolphins after 10 minutes and 3 hours of animal pursuit and capture. The expected results were that the cortisol concentrations would be markedly different and higher in the dolphins pursued for three hours versus the dolphins chased for only 10 minutes but they found no such difference (St. Aubin et al., 1996). In stress conditions, cortisol is known to increase when capture and handling are involved (St. Aubin and Geraci 1989, 1992; Thomson and Geraci, 1986). The opposite happens to T4, there is a decrease when capture and handling are involved. In a study using ACTH stimulation, Steller sea lions responded within 30 minutes with circulating cortisol concentrations, which were completely cleared from the body as fecal corticosterone 55 hours after stimulation (Mashburn and Atkinson, 2004). On the contrary, the ACTH study by Thomson and Geraci (1986) with bottlenose dolphins, there was little rise in cortisol one hour post-injection. There is an expected rise in cortisol one to two hours post ACTH injection and a decline to baseline values within four to five hours if no further stress stimulation occurred. In thyroxine stress tests, thyroxine did not show a marked decline until 20 hours post sampling (St. Aubin and Dierauf, 2001). This might have been due to the fact that thyroid hormones are modulated in stress to conserve energy reserves for more urgent metabolic needs.

Stress has been defined as the ability of an animal's sensory system to receive and interpret information about the surrounding environment, and the degree of positive and negative feedback that occurs during the response (Lovallo, 1997). Stress is generally defined as any stimulus; fear or pain, which disturbs or interferes with the normal physiological equilibrium of an organism (Flexner, et al., 1988). Four main categories

have been documented for stress in mammals; physiological, endocrinological, immunological and neurological. Within the four main stress type categories, there can be chronic or acute stress. A large suite of factors can cause a stress stimulus in an animal; however a stress response must be measured through subtle changes in one area of the four categories listed above. Not all stress is necessarily negative. Some stress can be positive and be seen as enrichment, such as the introduction of another animal to a colony or into a captive habitat, or a diet change (St. Aubin and Dierauf, 2001).

Acute stress can present as changes in behavior. Anxiety is the most often noted first outward indication of acute stress in an animal. "Stress swimming" or pacing in marine mammals, either in water or in pens, is a predominant sign of anxiety and thus, possibly acute stress. Acute stress is mostly diagnosed through blood chemistries. Certain parameters in the blood chemistries, such as creatine kinase and potassium, will show forms of exertional stress from capture and handling (St. Aubin and Dierauf, 2001). Acute stress can result in large spikes in baseline hormone blood parameters. Acute stress can be as serious as to bring on hypertension, hyperglycemia and metabolic acidosis leading to possible forms of pathological capture myopathy and in extreme case, respiratory and cardiac arrest (Spraker, 1993).

Chronic stress is usually negative, such as the effect of an oil spill on prey availability, contamination, or disease (St.Aubin et al., 1979). These are termed indicators of stress (Geraci and St. Aubin, 1990, Walker, 1995). Chronic stress may occur if stressors are frequent, repetitive or prolonged, without sufficient time for recovery in between stressful episodes. According to Dantzer and Mormeade (1995), there are three

response categories of chronic stress; habituation where the stress response decreases at each progressive stimulus, sensitization where the stress response increases at each progressive stimulus and desensitization where the stress response does not change at each progressive stimulus. In the chronic stress response, there is a pulsatile secretion of glucocorticoids, mainly determined by what form of stressor is present (Dantzer and Mormeade, 1995). Chronic stress can be associated with changes in reproductive status, organ function and constant infection, thus making it more significant than acute, or short term, stress. Cellular components can show signs of chronic stress. Lymphocytes will take longer to respond or fail to respond if the immune system is compromised. Adrenal glands will show histological or pathological evidence in necropsy of animal's suspected of chronic stress (St. Aubin and Dierauf, 2001). After stress or exposure to contaminants, animals may exhibit an immediate or acute response and/or a chronic response. Thermal stress is another stressor that pups encounter. Cortisol, like the thyroid hormones, can achieve a calorigenic effect and mobilize brown fat stores for thermoregulation (Engelhardt and Ferguson, 1980). All of these responses affect various homeostatic mechanisms in vertebrates, most significant being the immune system (Duffy et al., 1993). Defining evidence of stress, regardless of acute or chronic, is a high priority in marine mammal research.

Immunity and Hormones

Immunity is the ability of the defense mechanisms of the body to counteract detrimental effects of invading microorganisms, toxins, or other foreign bodies. Strong immune function is imperative for the survival of an animal. The immune function of an

animal can be suppressed by repeated attacks from microorganisms, toxins or foreign bodies causing disease, lowering the ability to fight incoming invaders. Immunosuppression is defined as "minimizing the fitness costs of an infection (via any means) after controlling for previous exposure to appropriate antigens" (Sheldon and Verhulst, 1996). Immunosuppression can be exacerbated by malnutrition, endocrine suppression and lowered development rate. Immunosuppression can be diagnosed using blood parameters, hormone levels and immunoglobulin profiles (Aldridge et al., 2001; Bossart et al., 2001).

There are two forms of immune response: humoral immune response and cell-mediated immune response. Cell-mediated immune response is brought about by leukocyte subpopulations (Aldridge et al., 2001). Three major subpopulations of leukocytes that may show immuno-compromise in animals are lymphocytes, neutrophils and eosinophils. These leukocyte subpopulations are easily quantified in diagnostic complete blood counts (CBC) in most laboratories, mostly by direct visual counts with microscopes. Lymphocytes are mononuclear leukocytes. In marine mammals, lymphocytes preside in lymphoid organs (spleen, lymph nodes, thymus and bone marrow) and circulate in low numbers in the blood (Bossart et al., 2001). T-lymphocyte activation in turn alerts monocytes and macrophages into immune response, mostly being responsible for the protection against intracellular microbes (Walker, 1995). Although many forms of lymphocytes exist, differential cell counts do not differentiate among them and they are counted as one large group by microscopic morphology (Tizard and Schubot, 2000). Viral and bacterial attacks elicit lymphocyte action (Ganong, 1979;

Tizard and Schubot, 2000). Proliferation of lymphocytes in response to a pathogen is necessary to the adaptive immune system (Aldridge et al., 2001). Eosinophils are circulating granulocytes, or polymorphonuclear leukocytes. They can amass in tissues, are generated in response to parasites, and can possess both parasiticidal and bactericidal properties (Clark and Kaplan, 1975; Ross et al., 1993; Bossart et al., 2001). Neutrophils are polymorphonuclear leukocytes and are formed in the bone marrow. There are two circulating activity groups of neutrophils; a circulating group and a group of cells inactive in the microvasculature. Neutrophils compose 60-75% of the leukocytes in carnivores (Tizard and Schubot, 2000). Neutrophils are activated by bactericidal infection, released by the bone marrow and the inactive group in the vasculature.

The second form of the immune response is the humoral immune system. The humoral system involves specific immunity attributable to antibodies (immunoglobulins), as opposed to the cell-mediated immune response brought about by leukocyte reaction to a pathogen (Aldridge et al., 2001; Walker, 1995). Immunocompetence can be measured by levels of circulating immunoglobulins (Tizard and Schubot, 2000). Neonates have a limited ability to express immunocompetence due to a low capacity to synthesize antibodies and thus must rely on passive immunity transferred from their mothers (Hall et al., 2002; Marquez et al., 2003). Immunoglobulins or, antibodies, are soluble, antigen-specific effector proteins that are associated with the humoral immune system that are shed by the lymphocyte B cells into the body fluids (Tizard and Schubot, 2000; Aldridge et al., 2001). These antibody proteins are made of heavy and light chains and shaped in a Y formation (Walker 1995). The importance of these antibodies is the way in which they

bind to foreign proteins. Certain binding properties, or epitopes, are the mechanism in which immunoglobulins can recognize and target invading, or foreign antigens. The measurement of circulating values for each specific immunoglobulin can aid in determining specific infectious compounds (Aldridge, et al., 2001).

IgG (gammaglobulin) and IgM (macroglobulin) are some of the major immunoglobulins found in circulation. IgG is the major immunoglobulin in humans and most species of mammals. It is formed in the plasma cells found in the spleen, lymph nodes, and bone marrow, and is present in body fluids and found on some mucosal surfaces. Plasma cells produce special proteins known as antibodies, or immunoglobulins. IgG escapes easily from blood vessels due to its small size and moves to target areas, especially inflamed tissues (Tizard and Schubot, 2000). IgG does cross the placenta, thus IgG is a maternal investment antibody (Walker, 1995; Tizard and Schubot, 2000). Hall et al., (2002) found that post-weaning circulating IgG levels may play a significant part in the survivability of grey seal pups from birth to age one year. The lower the concentration of circulating IgG in the pups, theoretically, the lower the probability of survival (Ross et al., 1993; Hall et al., 2002). Ross et al. (1993) noted that pups from birth to day 14 had increasing IgG levels. The theory from that study was that there was a low transfer of transplacental IgG and a high transfer rate through colostrum and milk during lactation. The caveat emphasized in many studies is that quantifying IgG levels is not a diagnosis nor does it give definite evidence to the level of immune system function in an animal. The measurement of immunogloblulins is merely another diagnostic tool used to aid in future baseline research in the field of immunocompetence

in animals (Ross et al., 1993; Aldridge et al., 2001; Hall et al., 2002). However, a detailed immunoglobulin profile would supply information regarding the immune function and capabilities of tested animals (Aldridge et al., 2001).

IgM is also formed in the plasma cells however, it does not easily escape from blood vessels due to large molecular size. IgM is the second highest immunoglobulin in concentration after IgG, but seems to not play a large part in the immune response at sites of tissue inflammation (Tizard and Schubot, 2000). IgM antibodies are the first to form when an invading protein or bacteria is detected and the major form to combat bacterial invasion in the primary immune response. It is active in the secondary immune response but it is masked by the activity of IgG (Tizard and Schubot, 2000). IgM does not cross the placenta and thus, must be synthesized by the fetal spleen or liver via antigenic recognition (Ganong, 1979; Walker, 1995). Both IgG and IgM cause opsonization. Opsonization is the coating of micro-organisms, namely bacteria, with antibody coating complements that enhance the uptake of the microbe by phagocytic cells. This action promotes the strength of the primary immune response (Walker, 1995; Tizard and Schubot, 2000). Immunoglobulins can be isolated from serum and purified if there are high circulating concentrations. Comparisons of immunoglobulin levels in serial serum samples can aid in determining if the animal in question may have immunosuppression, or immune function inhibition.

There are certain patterns in leukograms that may indicate long term stress, termed "stress leukograms", and these can be typified by lymphocytopenia or eosinopenia (Geraci and St. Aubin, 2001). High eosinophil numbers could mean a

response to parasitic infection (Tizard and Schubot, 2000; Aldridge et al., 2001). The immune system is sensitive and can be accurately measured and influenced by many internal and external stresses that affect marine mammals (see above section on Stress). Leukocyte subsets can aid in trying to determine the reason why the immune system may react to stress on an organism.

In a variety of species, substantial energetic costs are associated with immunological stress and maintenance of the immune system. It is likely that the magnitude of these costs is considerably large, being comparable to those involved with reproduction and growth. The cost of regulating the immune system during the response to challenge by a pathogen may push the animal beyond the level of body reserves needed for survival (Hall et al., 2002). Svensson et al. (1998) theorized from a study of the avian species, Blue tits (*Parus caeruleus*), that energy resource allocation during cold stress states the possibility that immunosuppression may avoid tissue damage, mediated through the production of stress hormones, caused by stress-activated immune defense mechanisms. Hall et al. (2002) and King et al. (1994) suggest that through other studies, grey seal pups' immune systems are fully mature at weaning and that IgG and IgM levels are at adult ranges. With values close to adult female ranges, these studies suggest that the pups should have the ability to mount an adequate humoral immune response if challenged (Hall et al., 2002).

Objectives

The two objectives of this project are to (1) establish baseline concentrations of total thyroxine (TT4) and cortisol in young harbor seals throughout the rehabilitation

process and to relate these levels to potential stressors and weaning, and compare them to wild harbor seal pups, and, (2) to assess immunocompetence of rehabilitated and wild harbor seals using leukocyte counts including lymphocytes and eosinophils, and correlating them with cortisol or TT_4 concentrations. The umbrella question is; what potential stressors are rehabilitated seals being exposed to and how do their endocrine and immune responses compare to their free-ranging counterparts of the theoretical same age? The hypothesis is that the free-ranging harbor seal pups are different from harbor seal pups in rehabilitation in terms of morphometrics, hormone levels and certain forms of cellular immune function.

This project will use our ability to monitor two metabolic hormones in combination with standard morphometrics and determine how they lend to the overall health of neonate harbor seal pups in rehabilitation settings. This study will also determine baseline concentrations of total thyroxine and cortisol of captive seals, housed in a stable environment with regular and balanced diets, and of free-ranging harbor seal pups. In doing so, I may be able to determine if the groups of harbor seals admitted to rehabilitation settings are comparable to groups of harbor seal pups in chosen populations in California and Alaska. Long-term monitoring and rehabilitation values of these hormones will aid in forming a working baseline for harbor seal pups. With the establishment of a baseline of metabolic hormones at admittance and followed through the rehabilitation process, it will allow for assessment and treatment of pups more efficiently. How marine mammals respond to stress, whether in captivity, rehabilitation or in the wild, will provide a better understanding of how humans and marine mammals

can co-exist. Further research is needed to determine how to develop less stressful environments for marine mammals, and how to lower the immunological and behavioral detriments of stress.

The information gained in this study will enable assessment of three groups of animals; those that live in a stable environment (captivity), with those that experience the natural environment (rehabilitated and wild, free-ranging pups). The ability of harbor seals to adapt to a changing environment is essential to the recovery and stabilization of their populations. This study will examine cortisol and TT_4 levels in neonatal harbor seals in two rehabilitation facilities, the Alaska SeaLife Center in Seward, Alaska and the Marine Mammal Center in Sausalito, California. The data collected for TT4 and cortisol will be used to form baseline values on pups that are admitted into these two rehabilitation facilities. Results from the hormone analysis will also be correlated to variables such as; weaning changes in hormones, age, and morphometric changes. Results from leukocytes (lymphocytes and eosinophils), and WBC (white blood cell) counts from the hematology differentials will be correlated as well. The importance of establishing a baseline in rehabilitation settings is to determine health status of animals coming into the facility. Ultimately, guidelines on thyroxine and cortisol concentrations of "normal" and "diseased" states in neonates would be extremely important in facilitating expedient medical regimes. Baseline information on endocrine and immune system function in harbor seals is one approach to further the investigation of immunocompetence of the species.

Chapter 2 Comparison of Two Metabolic Stress Hormones and Two Leukocyte Subsets in Rehabilitated and Free-Ranging Harbor Seal Pups

Introduction

Harbor seal pups are precocious at birth (Dierauf et al., 1986; Riedman, 1990; Muelbert and Bowen, 1993) and they typically wean anywhere from 4 to 6 weeks. As precocial as harbor seals pups are at birth, if they become separated from their mothers at too early an age, they are in danger of malnutrition, disease, or increased susceptibility from predation (Riedman, 1990). Neonates are the age class with the lowest survivorship. If a neonate is disturbed on the beach or separated from its mother, the likelihood of it surviving declines. Usually, these pups are more liable to develop health problems due to not receiving enough or any immunity or colostrum from maternal milk. Neonates also have to constantly regulate their body temperature. Proper fat and protein deposition is crucial for pup survival. Pups are born with a layer of insulting brown fat and put on extensive weight, mainly as blubber during the lactation period, but may still exhibit problems with thermoregulation, mainly hypothermia (Riedman, 1990). Due to low amounts of body fat, especially if no nursing has occurred, the core temperature of the pup can drop below a critical level, or the thermal neutral zone (TNZ), exposing the animal to hypothermia (Schmidt-Nielsen, 1997). High amounts of energy are expended to try to keep within the TNZ, post-utero. Blubber is the most efficient form of insulation for a marine mammal when dealing with both cold water and exposure to cold air.

Stress has been defined as the ability of an animal's sensory system to receive and interpret information about the surrounding environment, and the degree of positive and

negative feedback that occurs during the response (Lovallo, 1997). Stressors may include nutritional stress from inadequate quantity or quality of food, feeding changes (i.e., weaning), maternal abandonment, disease processes, noise, or contact with other species. Many factors can cause a stress response in an animal; however, a stress response must be measured through subtle changes in one area of four indicators of stress; physiological, endocrinological, immunological or neurological (Geraci and St. Aubin, 1990). Within the four main stress response categories, there can be two further divisions; chronic or acute. Chronic stress is defined as a stress state that is prolonged over time, while, acute stress is defined as a state that rapidly develops but is short-lived (Walker, 1995; Mashburn and Atkinson, 2004). Chronic stress may occur if stressors are frequent or repetitive. Chronic stress can be associated with changes in reproductive status, organ function and persistent infection, thus making it more significant than acute stress. Immune function can sometimes be compromised by high levels of stress. Acute stress is mostly diagnosed through blood chemistries. Acute stress can show large spikes in baseline hormones and blood parameters such as in plasma enzymes and erythrocyte ranges (St. Aubin et al., 1979; Morgan et al., 1998).

Strong immune function is imperative for the survival of an animal. Immunosuppression can be exacerbated by forms of malnutrition, endocrine suppression or exacerbation, lowered development rate and poor fecundity (Tizard, 1992; Aldridge et al., 2001). Immunosuppression can also be diagnosed using blood parameters, hormone concentrations and immunoglobulin profiles (Aldridge et al., 2001; Bossart et al., 2001). There are two forms of immune response; humoral immune response and cell-mediated

immune response. Cell-mediated immune response is brought about by leukocyte subpopulations (Aldridge et al., 2001). Two major subpopulations of leukocytes that may show immunocompromise in animals are lymphocytes and eosinophils. Changes in these leukocyte subpopulations are easily measured in routine diagnostic blood tests. Eosinophils can amass in tissues and are generated in response to parasites and can possess both parasiticidal and bactericidal properties (Clark and Kaplan, 1975; Ross et al., 1993; Bossart et al., 2001). In marine mammals, lymphocytes preside in lymphoid organs (spleen, lymph nodes, thymus and bone marrow) and normally have low circulating numbers in the blood (Bossart et al., 2001). Viral and bacterial attacks elicit lymphocyte action (Ganong, 1979; Tizard and Schubot, 2000). Proliferation of lymphocytes in response to a pathogen is necessary to the adaptive immune system (Aldridge et al., 2001).

The endocrine components of the stress response include the primary response of producing the hypothalamus and pituitary peptides, CRF and ACTH, catecholamines, epinephrine and the secondary endocrine components, which includes the synthesis of glucocorticoids such as cortisol, responding in a cascading fashion from the primary endocrine components. This cascading response leads to tertiary components, among them, thyroxine (Dunn, 1995; Lovallo, 1997; Breazile, 1988). The link between the hypothalamus, the pituitary gland and the adrenal glands is called the H-P-A axis. It is a feedback regulation loop in which the stressor stimulates the adrenal cortex to synthesize and release cortisol. Adenocorticotropic hormone (ACTH) stimulates the hypothalamus to release CRF (corticotropin-releasing factor), which in turn stimulates the pituitary to

produce ACTH. The hypothalamus secretes more CRF in response to stimuli (stress, for example) and lowered cortisol concentrations. This cascade increases the ACTH production and subsequent increases in cortisol production. This system is one of the most important endocrine regulation systems (Hines, 2004). Chronic stress is documented to cause sustained activation of the HPA axis producing sustained secretions of glucocorticoids (St. Aubin and Dierauf, 2001).

The most complicated aspect of studying a stress response in an animal is the ability to determine the difference between a true stress state versus an unstressed, or baseline state. Many diagnostic techniques (capture, handling, sampling, blood draws, confinement) have the ability to bring about a stress state in an animal. In two species of cetaceans under stress, cortisol increased when capture and handling were involved. In the same studies, the opposite happened to thyroxine: it decreased when capture and handling were involved (St. Aubin and Geraci, 1987; Thomson and Geraci, 1986). This might have been due to the fact that thyroid hormones are modulated in stress to conserve energy reserves for more urgent metabolic needs (St. Aubin and Dierauf, 2001).

Cortisol is the dominant circulating glucocorticoid in all marine mammals (Ortiz and Worthy, 2000). Cortisol is a glucocorticoid steroid, which is released directly from the adrenal cortex, stimulated by the anterior pituitary gland. The adrenal cortex is well developed in neonatal harbor seals, partially explaining precociousness in the species (St. Aubin, 2001). Cortisol acts to maintain a level of homeostasis in the body to aid against environmental pressures (St. Aubin et al., 1996). Cortisol acts upon protein, lipid and carbohydrate mobilization and metabolism, aids in varied adaptations of the immune

reaction to a number of distresses, such as allowing T-cells to self-regulate and producing several enzymes for pathogenic and anti-inflammatory response, thus limiting cell and tissue damage by doing so (Eckert et al.1988; Weber, 1997). Glucocorticoids, such as cortisol, stimulate immune function by decreasing their concentration in serum (Weber, 1997). Cortisol is an indicator of health status in an animal due to levels rising during times of stress (Oki, 2001). An increase in cortisol secretion causes the breakdown of muscle protein into amino acids and the amino acids are metabolized in the liver into glucose for energy though a process called gluconeogenesis. At the same time, cortisol also causes fat to breakdown into fatty acids used in the muscles for energy. After enough fuel is provided, cortisol secretion declines providing negative feedback to the pituitary such that ACTH declines (Stoppler, 2004). When faced with stressful situations these feedback loops give the body and brain adequate fuel to deal with the stress. Cortisol is suggested to increase within 25 to 30 minutes after exposure to the initial stressor (St. Aubin, 2001).

Thyroxine (T4) is one of two thyroid hormones secreted via the thyroid glands, stimulated by thyroid stimulating hormone (TSH). Thyroxine aids in influencing aspects of reproduction, growth, metabolism and development of the immune system (Hall et al., 1998, Woldstad et al. 1999, St. Aubin and Geraci, 1987). Thyroxine can be found as free or bound. Free T4 is thyroxine that is circulating in the bloodstream, not bound to a carrier protein, such as TBG (thyroid binding globulin). Bound T4 is thyroxine that is bound to a carrier protein. Total T4 is the total amount of thyroxine found circulating, both bound and free. During periods of distress, thyroid hormone concentrations decrease

as part of a larger feedback system to aid in conserving energy reserves (St. Aubin and Geraci, 1987). Findings from studies of beluga whales brought into captivity (St. Aubin and Geraci, 1987) showed that the increased glucocorticoid component of the stress response realigns to conserve thyroid hormone balance. Due to the negative feedback loop present in the cortisol-thyroid hormone relationship, subsequent decreases in circulating cortisol concentrations elicited a compensatory increase in thyroid concentrations (St. Aubin and Geraci, 1987). Another thyroid hormone, T3, or triiodothyronine, is formed from the conversion of T4. Most measurements of thyroid hormones, such as thyroxine, in all ages of an animal are remarkably constant, though this applies to T3 moreso than T4 (Hall et al., 1998). However, marine mammal neonates may differ in this case. Amoroso et al. (1965) found that common and grey seal neonates exhibited variable to large neonatal thyroid gland size, it then began to decrease shortly after birth. Engelhardt and Ferguson (1979) found that harp seal newborns had the highest T4 levels of all age classes in the species, followed by a rapid decline, almost a 40% decrease at 3 weeks of age. This seems to indicate a calorigenic function to mobilize brown fat and glycogen stores for thermoregulatory processes (Blix et al., 1979, Leatherland and Ronald, 1979; Woldstad and Jenessen, 1999). Hall et al. (1998) concluded that grey seal pup thyroxine levels were higher in pre-weaned pups in comparison to adults but not higher to post-weaned pups. Adult levels of thyroxine were reached by the pups at approximately 18 days of age.

The purpose of this study was to monitor two metabolic hormones and two leukocyte subset parameters to measure immune function in combination with standard

morphometrics, and relate their role to the overall health of neonate harbor seal pups in rehabilitation settings and in the wild. Cell-mediated immunity was quantified using two clinically monitored leukocyte populations: eosinophils and lymphocytes. With long term monitoring of wild, free-ranging seals and those undergoing rehabilitation, these hormones and leukocytes will aid in forming a working baseline to which potential general health conclusions may be related. The questions are; (1) do anthropogenic stressors alter metabolic hormone concentrations in rehabilitated pups more than stressors found in wild populations of harbor seal pups, and (2) can changes in metabolic hormones and leukocytes be detected in response to interventions that are undertaken as part of the rehabilitating of harbor seal pups?

Methods

Animals and Sample Acquisition

Two groups of seals were used in this study; 1) seal pups admitted for rehabilitation; 2) free-ranging seal pups. Pups undergoing rehabilitation included pre-weaned and post-weaned pups, up to the maximum age of 8 months, from the Alaska SeaLife Center (ASLC) rehabilitation department (n=20) and the Marine Mammal Center (TMMC) in California (n=12). All harbor seals admitted under the age of approximately 6 months in Alaska were included, and any extra serum generously donated from The Marine Mammal Center's routine diagnostic and clinical veterinary procedures from pups under the approximate age of 8 months. Blood was normally drawn from animals in rehabilitation every 10 to 14 days. Blood was normally drawn from the extradural intervertebral vein with 21G x 1.5 inch spinal needles. Average restraint time on animals

at ASLC was less than 7 minutes. TMMC animals were restrained for less than 20 minutes. The pups were minimally physically restrained without chemical immobilization. Chemical restraint was only used in older post-weaning seal pups, if blood collection was accompanied with satellite tagging procedures (n=4). This was normally the last sample collected prior to release. If this was the case, a mass based dose (0.055mg/kg) of Torbugesic (Butorphanol tartate) was used. The serum from all blood draws was frozen at –80 degrees C until analysis. In some cases when serum was unavailable, plasma was validated and used *in lieu* of serum (Oki and Atkinson, 2004). Blood samples from wild harbor seal pups (n=60) was collected under Alaska Fish and Game permits #1000 and #358-1585. These samples were collected from the years 1997 to 2000 on Tugidak Island and generously donated by Drs. Steven Trumble and Michael Castellini at the University of Alaska, Fairbanks. Pups sampled from Tugidak Island were all deemed post-wean due to time of year and lack of maternal presence.

Radioimmunoassay Analysis

Hormone concentrations were measured using solid phase radioimmunoassay kits specific for cortisol or total thyroxine (TT4) (Diagnostic Products Corporation (DPC), Los Angeles, CA)). Mean non-specific binding for TT4 and cortisol were 1.10% and 1.01%, respectively. Quality control indicators, provided by DPC, were included in each assay. Pooled samples from young captive harbor seals were run in each assay to determine and track interassay variation. Intraassay variation was less than 5% and interassay variation for all assays was less than 10%. Sensitivity of the assays for TT4 and cortisol were 0.33ng/tube and 4.5ng/tube, respectively (DPC standards). The standard

curves of each assay were log-logit transformed, enabling extrapolation of sample concentrations (Rodbard, 1974). Parallelism was determined using a 25%, 50%, 100% and 200% of pooled sera added to the standard curves for both cortisol and TT4 to ensure that these curves were parallel to the standard curves without serum.

Morphometrics, Aging Techniques and Weaning

Morphometrics were taken at the time of each blood collection for all animals and included of standard length (cm), weight (kg), axillary girth, hip and maximum girth measurements (cm). The free-ranging pups were weighed and measured at capture. The pups undergoing rehabilitation were weighed every one to two days upon arrival and shortly after admittance, however, after a pup had been stabilized, they were weighed and measured only at sample collection (for blood and morphometrics), or less often. Not all measurements were taken each time due to time constraints or the behavior of the animal.

Age was estimated at initial physical examination of the animal upon admittance to the rehab center. Age indicators were status of dentition, presence of an umbilicus and the state of the umbilicus. Animals with an umbilicus were usually neonate animals, 1 week or younger. Coat condition referenced whether or not the animal still had any form of lanugo, or neonatal coat, which is normally shed *in utero*. Animals with no dentition, lanugo present, and a pink or even bloody umbilicus present were the youngest animals seen and were presumed premature. Older pups had shed their umbilicus, possessed a new adult looking pelage and had erupting teeth.

Weaning during rehabilitation consisted of taking the pup off of the fat-rich fabricated formula that had been administered via stomach tube onto a solid fish diet.

Formula was made up of a mix of Zoologic 30/55 (PetAg, IL), a powdered animal milk matrix fabricator, with water and salmon oil added to make a milk matrix that closer mimicked the fat and nutrient content of wild harbor seal milk. Weaning time was determined by the weight, feces status (lack of dehydration), age, dentition and overall general health of the pup. Harbor seals are typically weaned anywhere from 4-6 weeks of age in the wild, and rehabilitation centers try to mimic this time range, depending on the health of the pup. Some pups were not as easily weaned and continued on an interim diet of formula and fish, approximately 50% or a 2:2 ratio of each feed type, past the normal documented weaning age. Pups were fed on a 10% body weight scale per day. Rehabilitation centers sometimes do a "soft wean" where the formula is decreased along with an increase in fish into the diet. This is to ensure that the pup does not lose weight or water balance in this time of changing diet and metabolic status.

The weaning process usually began by introducing dead fish after stomach gavage. If there was interest, the process of "fish schooling" the pup began; to help the pup correlate fish as food. After a set amount of time, dependent on age, behavior and health status of the pup, force-feeding of fish would be a supplement to formula. Force-feeding consists of some sort of restraint to the pup and gently guiding whole small fish down the pup's esophagus and past the gag reflex point. Formula was weaned out of the diet and fish was the only source of food. The balance between the weaning of the formula to fish was delicate in that the pup's metabolism would completely change in a matter of a few days. In the wild, the mothers typically leave the pup to forage and find fish on their own after four to six weeks.

Statistics

Mean and standard error were calculated: 1) per hormone per animal; 2) per sampling period and 3) per group of animals to be compared (wild or rehabilitating), 4) per leukocyte subset concentration per animal. T-tests were run in SigmaStat to test the difference of the means in the two groups of animals. A one-way ANOVA was run on the three groups of seals (pre-, post-weaned and wild) to detect differences among the mean hormone concentrations, masses and leukocyte subset concentrations in the pup groups. Standard linear regressions were run to determine relationships between age and mass for the three groups of seals. All morphometrics or hormone concentrations over time were plotted with weaning period range and any other significant events (age, morphometric changes and some disease states). Due to large weaning period ranges, the TMMC pups were excluded from the weaning date and metabolic hormone correlations for statistical accuracy. However, TMMC pups were used in all morphometric and raw metabolic hormone comparisons where weaning periods were not a factor in determining results.

The sample sizes for the lymphocyte and eosinophil comparisons were different because not all pups had recorded values for either of the cells, or because of fewer than 3 data points per seal pup. Animals were not further divided into categories based on death or release (n=3, dead) because most animals that died in rehabilitation did not have enough data points to be used in the statistical comparisons. "High" and "low" leukocyte determination was established by looking at every data point per animal and deciphering if the highest or lowest value in the serial data was to be found at the point of weaning or the point of release: the data point that correlated with either of those designations.

Results

Hormone concentrations

The pre-weaned harbor seal pups undergoing rehabilitation had the highest mean and standard error cortisol concentrations (Table 1). The post weaned pups exhibited the lowest mean cortisol concentration of all three groups. There were some indications that cortisol spiked 2-5 days prior to death in rehabilitation pups (n=3), although the sample size was small. Cortisol concentrations were significantly different ($p=0.049$) between the wild pups and the post-weaned pups and ($p=0.003$) when pre-weaned, post-weaned and wild pups were compared using a one-way ANOVA test. The wild pups had the highest TT4 concentrations of all three groups, however the pre-weaned pups had the highest standard error (Table 1). TT4 levels were depressed prior to successful pup releases (n=20). TT4 was significantly different between the pre-weaned rehabilitation pups and the wild pup samples ($p=0.017$). Sex did not influence hormone levels. This is most likely because these animals were far below sexual maturity.

Weaning and Age Range Data

Weaning had opposite effects on cortisol and TT4 levels in the rehabilitation pups. Cortisol showed decreases (n=11, 44%) directly after weaning in each individual of the ASLC seals when graphed using Julian days on the x-axis from the period from admittance through weaning, to release, or death. Events that occurred along the x-axis on the Julian day graphs highlight different stressors the animal experienced throughout the rehabilitation process at ASLC and how the two metabolic hormones reacted. Figure

2-1 shows all animals in rehabilitation for both facilities and each animal's weaning duration. Eight of the largest time ranges for weaning (beyond 15 days) were seen in animals from The Marine Mammal Center (N=32, n=8). Pups from TMMC had larger and more variable weaning ranges (3-27 days; Figure 2-1). However, the range of weaning and hormone ranges do not necessarily correlate to each other. After reviewing each pup's individual hormone patterns, pups with long weaning periods do not always show a negative relationship in terms of high cortisol during these time ranges (Figures 2-2, 2-3). These graphs show two animals; one with a relatively short weaning period of 6 days (Reba, Figure 2-2) and an animal with a relatively long weaning period of 31 days (Half Pint, Figure 2-3). Even with the differing time ranges in weaning, both animals show decreased cortisol and TT4 at the last sampling prior to release.

TT4 increased (N=32, n=20, 63%) directly after the onset of weaning in rehabilitated pups. As the pup aged in rehabilitation and was released, cortisol decreased at the last bleed prior to release (n=16). TT4 showed mixed results, most pups showed decreases prior to release (n=20), but there were still almost 38% of the released animals that showed increased TT4 prior to the release.

Leukocytes

Pups from both facilities were compared by charting the two leukocyte groups in terms of; (1) the bleed directly after weaning, and (2) the last bleed before the pup was released from rehabilitation (Table 2). Weaning had mixed effects on pups in rehabilitation in terms of both eosinophil and lymphocyte counts (Table 2-2). Pre-weaned and post-weaned rehabilitation pup mean lymphocyte percentages were closer in value

and significantly lower than those of the wild pups, as a whole and as compared to the both the pre weaned and post weaned rehabilitation pup groups (Table 2-2). Wild pups had a higher lymphocyte count compared to the rehabilitation pups. However, pre-weaned and wild pups were more closely comparable in eosinophil range than with pre-weaned and post-weaned rehabilitated pups. There was quite a large difference, in raw data, between pre-weaned and post-weaned rehabilitation pup mean eosinophil absolute counts. The absolute leukocyte values would normally be a secondary diagnostic tool to compare leukocyte percentages to see if there was a medical concern. The post weaned pups show a larger mean of both lymphocyte and eosinophil absolute counts. However, after calculating the means and standard errors of both lymphocytes and eosinophils for the rehabilitation pups, pre and post weaned, the standard errors are too large to show a significant difference between groups.

The highest percentage of lymphocytes found was in the "last bleed" category (Table 2). The lowest percentage of lymphocytes was found in the "weaning" category. Conversely, one of the highest percentages for the eosinophil categories was in the "weaning" category of animals. However, the equally high percentage of eosinophils at last bleeds is in agreement with the last bleed group of animals with high lymphocyte percentages. The smallest percentage for eosinophils is in the low at weaning category. The high at weaning and high at last bleed category percentages were equal (58%) in the eosinophil groups while the low at weaning and low at last bleeds category percentages (47%) were equal to each other in the lymphocyte groups. A small percentage of animals exhibited both high and low counts of either leukocytes at both weaning and last bleed

from both facilities. For example, the largest percentage, 47% of rehabilitated pups, had low lymphocyte counts at both weaning and last bleed categories. The percentages calculated for groups of animals sharing the highest or lowest range of each leukocyte group was negligible.

Discussion

A comparison between rehabilitation and free-ranging harbor seals was designed utilizing metabolic hormones; cortisol and TT4, and cell-mediated immunity factors, lymphocytes and eosinophils. Wild harbor seal pups had the highest concentration of total thyroxine (TT4) of the three groups of pups compared while the post weaned rehabilitation pups show the lowest concentration (Table 2-1). The reason for these results is likely that pups in the wild expend more energy for maintenance in the natural environment compared to pups in rehabilitation. The pups in the wild must forage for their food to stay alive, escape from predators, and stay in a thermal neutral zone to maintain homeostasis. All of these challenges might result in elevated circulating concentration of TT4 for the wild pups. This was surprising due to literature stating that pre weaned pups (neonates) usually have the highest level of thyroid hormones due to the hyperactivity of the thyroid gland, especially in harbor seals (Harrison et al., 1962; Amoroso et al., 1965; Haulena et al., 1998; Woldstad and Jenessen, 1999). However, it is also possible that some rehabilitation pups might be euthyroid or hypothyroid. The post weaned pups were expected to be lower based on literature that states circulating thyroid hormones usually decline during the first few weeks of life (St. Aubin, 2001). Heat is a by-product of gluconeogenesis and thus, influenced by cortisol as well as the thyroid

hormones (Oki and Atkinson, 2004). The more circulating cortisol, the more heat is produced to maintain body core temperatures. This is an adaptation in pups living in a cold environment where heat via food intake may be limited. Thyroxine is the dominant metabolic hormone that controls thermoregulation. TT4 helps to increase the rate of metabolic heat produced by increasing the rate of glucose oxidation by breaking bonds in ATP (Nelson, 1995; Oki and Atkinson, 2004). This may account for the higher concentrations of TT4 in the wild pup samples. Higher concentrations of TT4 compared to both pre- and post-weaned rehabilitated pups, taking into account the use of these two hormones in the metabolic system, the results support the idea that the thyroid concentrations are higher when compared to the rehabilitation pups due to higher metabolic energy needs and higher thermoregulatory needs.

The wild pups were larger in mass and length at the same post-weaning age as the rehabilitation pups. There may be many reasons for this, one being the efficiency and complete sustenance of maternal investment. Harbor seal milk is approximately 45-48% milk fat throughout lactation with a higher percentage of fat early in lactation, approximately the first and second week (Boness and Bowen, 1996; Riedman, 1990). Fabricated milk substitute, even when supplemented with high fat salmon oil, is only approximately 32-35% fat. The increased fat in the maternal milk along with other nutrients that milk replacement formula can not mimic likely leads to reduced growth rates in rehabilitated pups. The other theory is that the rehabilitation harbor seal pups are often sick when admitted to rehabilitation centers, normally including moderate to severe

emaciation and dehydration resulting from malnutrition and abandonment, thus predisposing them to lowered growth rates than their wild con-specifics.

Wild harbor seal pups had the highest mean lymphocyte percentages when compared to rehabilitation pups. There may a connection between the increased cortisol and decreased total thyroxine levels and the higher lymphocyte ranges, since when cortisol is found in higher concentrations, TT4 is conserved in a feedback loop for saving metabolic energy. In addition, immune systems in marine mammals are known to show sensitivity to stress-related hormonal changes (St. Aubin and Dierauf, 2001). Both cortisol and thyroid hormones, including thyroxine, aid in maintaining the immune system (Eckert et al., 1998; Hall et al., 1998). If the thyroid hormone concentrations are elevated for various reasons such as thermoregulation and other metabolic demands, it may be possible to attribute the higher lymphocyte ranges to the higher hormone concentrations. In other terms, if TT4 is increased metabolically, there may be a compensatory increase in all forms of the immune response, whether a pathogen was present or not. However, the wild pups' eosinophil ranges were not the highest of the three categories of pups. One main difference between the two leukocytes is that lymphocytes respond to a viral load whereas eosinophils usually do not. A possible explanation for the post weaned pups to show a higher concentration of eosinophils might be due to the life cycle of many parasites. The average time it takes for many parasites found in seals to mature is between 4-6 weeks. This is approximately the time the pups are weaning. Since eosinophils increase with parasite load, it is possible that these pups

have parasite loads due to the maturing parasites that were not detectable via the immune system in younger animals (Brown, personal communication).

Pre-weaned rehabilitating harbor seal pups exhibited the lowest levels of both leukocyte types of all three groups compared. The pre-weaned pups have the highest cortisol concentrations of all three groups of pups compared. The pre-wean pups also had a higher concentration of total thyroxine than the post-wean pups. This supports the concept in the literature that thyroxine is related to metabolic heat and thermoregulation demands (Blix, 1979). Neonate pups, some even premature, have many metabolic challenges to deal with in the first two months of life. A blubber layer has been established for the pups; however the layer is just not as established or thick as it needs to be for efficient thermoregulation (Riedman, 1990). Blubber is the best source of thermoregulatory material for these young pups. All organ systems are still developing and the endocrine system must regulate all hormone concentrations in the body. The immune system may be weak due to lack of maternal immunity or a disease state.

Cortisol is a "stress" hormone. It is reasonable to expect a developing animal's system, for example that of pre-weaned pups, to show higher cortisol concentrations than post-weaned and wild pups. Post-weaned rehabilitation pups had the lowest TT4 levels of the three groups compared in this study. This might be because these animals do not need to exert extra energy for foraging or thermoregulation. Animals in rehabilitation are fed on a consistent timeframe, a diet at least 8-10% of their weight per day. Though these pups may be in outside pens and in pools with other competing conspecifics, the pressure on the metabolic hormone feedback loop is not strong. Pressures that could alter the

hormone feedback loop would be predator stress or energy needed long foraging trips. As expected, the mean mass of the pre-wean pups is significantly less than the wild or post-wean rehabilitation pups. However, the post-weaned rehabilitation pups showed a lower mean body mass and length when compared to wild pups of the supposed same age. Again, this may be due to the outcome of a stunted growth rate because of lack of maternal investment, not acquiring proper fat (blubber), or having a disease state present.

The question whether stress is a factor for rehabilitating seal pups is strongly debated in the scientific community. Stress is also one of the hardest states to try to quantify in research. However, examining these results from five consecutive years of rehabilitation pup sampling and three years of wild pup sampling from the same location shows that pre-weaned rehabilitating pups have higher cortisol concentrations, which is not surprising. The wild harbor seal pups showed elevated levels of the thyroid hormones and one of the leukocyte populations, reflecting the challenges of being a pup in the natural environment. This information is advantageous for the scientific validity of marine mammal rehabilitation centers in that the results do not show an undue amount of chronic stress states on the hormone levels of harbor seal pups admitted and treated in rehabilitation centers.

Table 2-1: Hormones and Morphometrics of Rehabilitated, Free-Ranging and Captive Harbor Seals

		Cortisol (ng/ml)		TT4 (ng/ml)		Weight (kg)		Length (cm)	
	n	mean	SE	mean	SE	mean	SE	mean	SE
Pre-wean rehab pups	32	16.4	1.83	3.1	0.31	10.2	0.29	65.7	4.59
Post-wean rehab pups	32	11.5	0.90	2.8	0.21	19.6	0.62	83.2	2.91
Adult captives	7	12.2	0.50	3.0	0.10	63.4	0.9	137.6	1.01
Free-ranging pups	59	13.1	0.52	3.8	0.14	26.9	0.70	94.5	0.82

Table 2-2: Percent and Absolute Leukocyte Counts of Rehabilitated and Free-Ranging Harbor Seal Pups

		Lymphocytes (%)			Eosinophils (%)			Absolute Lymphocytes			Absolute Eosinophils	
	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
Pre-wean rehab pups	32	15.5	1.5	19	1.6	0.3	17	174.2	13.1	16	22.8	3.8
Post-wean rehab pups	32	15.9	1.5	19	4.0	0.5	17	210.9	16.6	16	57.2	11.8
Free- ranging pups	59	26.0	1.4	59	2.9	0.3						

Table 2-3: Harbor Seal Pups' Leukocyte Percentages at Weaning and End of Rehabilitation.

Lymphocytes	**N**	**n**	**%**
High at wean	32	13	41
Low at wean	32	15	47
High at last bleed	32	16	50
Low at last bleed	32	11	47
Eosinophils			
High at wean	19	11	58
Low at wean	19	4	21
High at last bleed	19	11	58
Low at last bleed	19	6	32

* N=whole group sampled, n= number of animals exhibiting high or low leukocyte percentages

"High" and "low" determination was established by looking at every data point, per animal and deciphering if the highest or lowest value in the serial data was to be found at the point of wean or the point of release: the data point that correlated with either of those designations.

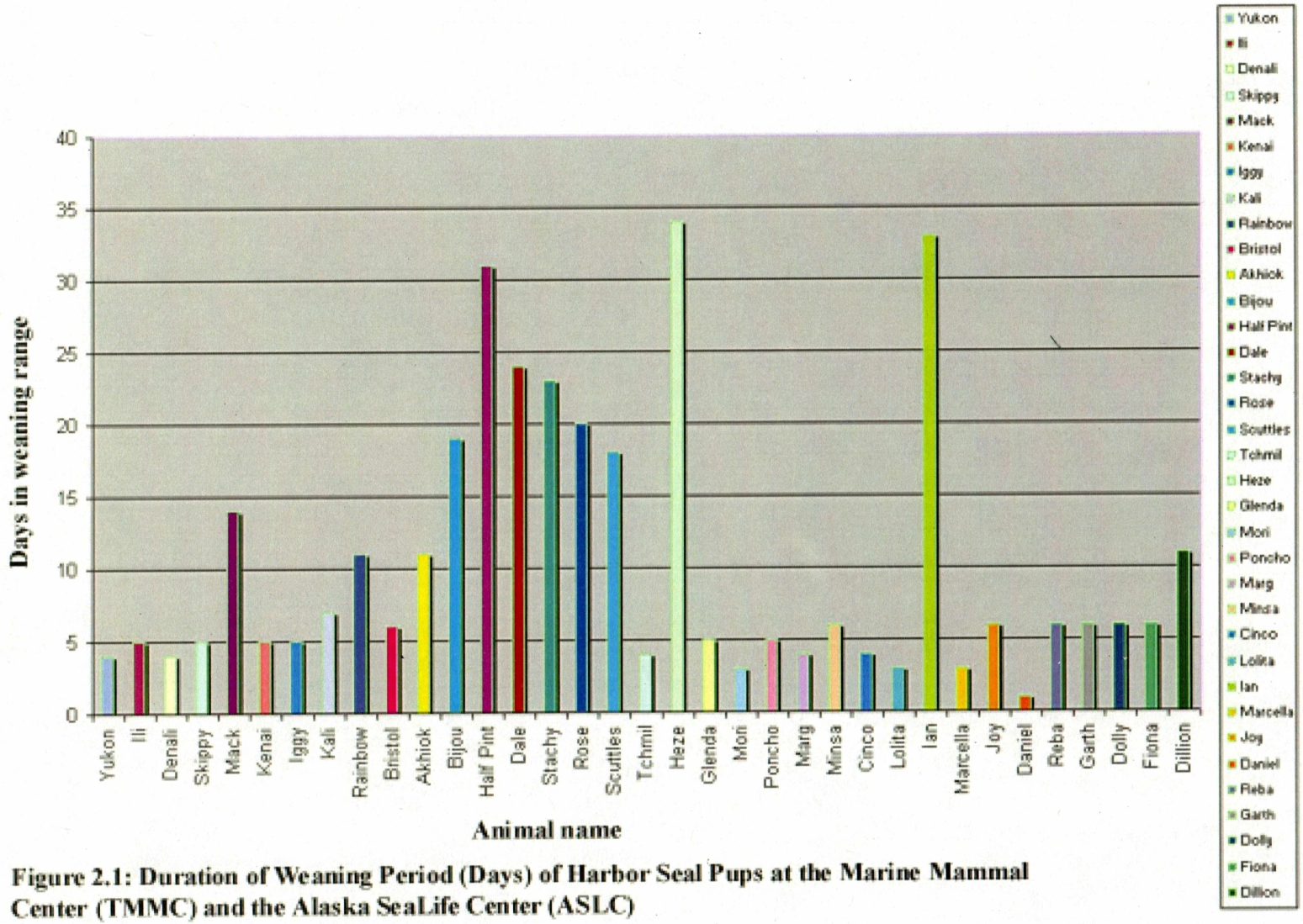

Figure 2.1: Duration of Weaning Period (Days) of Harbor Seal Pups at the Marine Mammal Center (TMMC) and the Alaska SeaLife Center (ASLC)

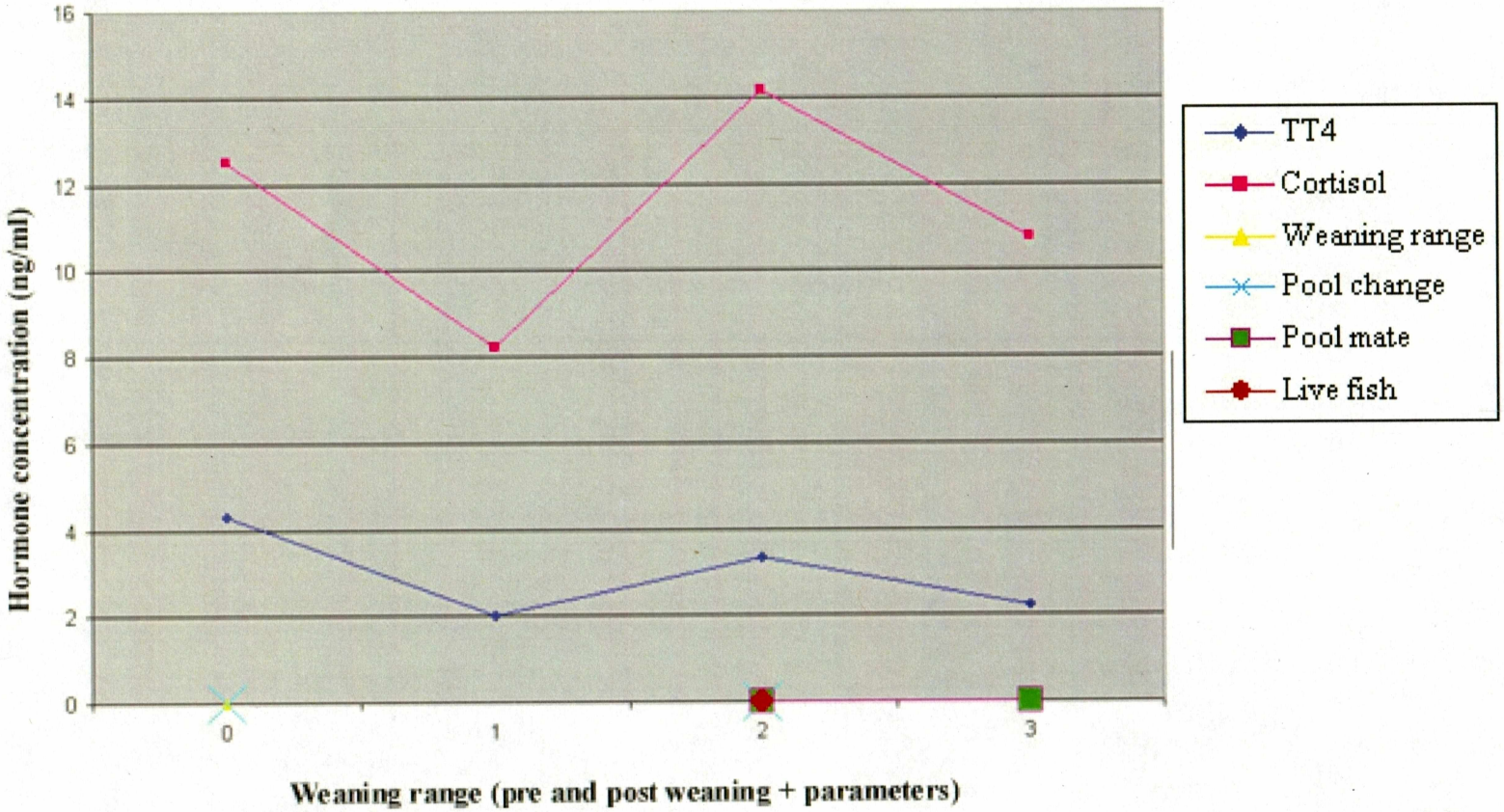

Figure 2-2: Rehabilitated Harbor Seal "PV0203 Reba" Hormone and Weaning Range Parameters at the Alaska SeaLife Center (ASLC)

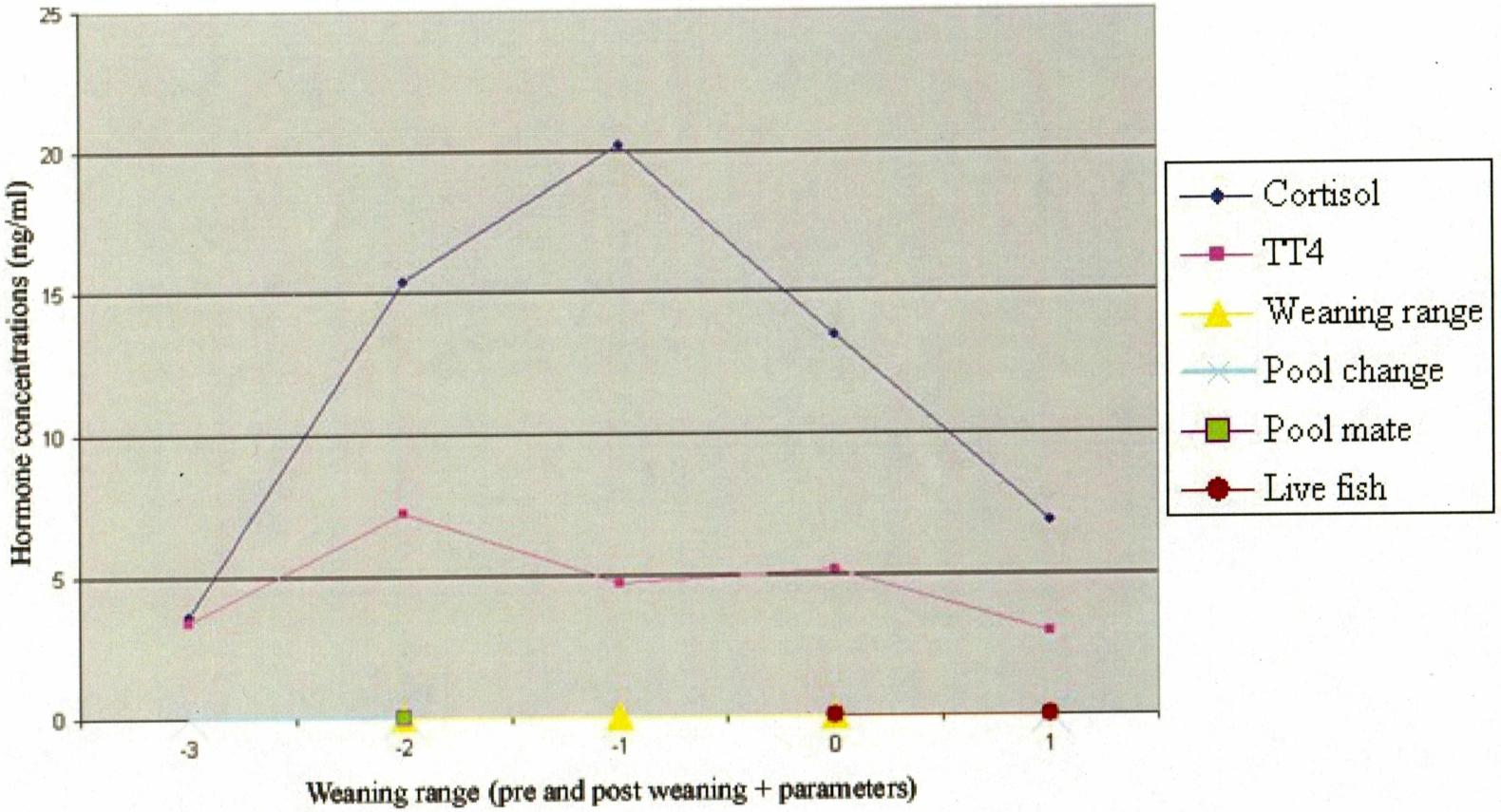

Figure 2-3: Rehabilitated Harbor Seal "HS 1396 Half Pint" Hormone and Weaning Range Parameters at the Marine Mammal Center (TMMC)

Chapter 3 Captive Harbor Seal Hormone Parameters

Introduction

Captive harbor seals offer a wide range of research opportunities. Normally fed a predictable and quantifiable diet, housed in stable environmental conditions and trained using operant conditioning techniques, captive seals are ideal as research subjects. More stable factors involving climate control, prey availability, temperature regulation and metabolic output in terms of exercise are aspects of captivity that help researchers to establish seasonal baseline data on animals in permanent captive facilities, as well as give insight into what free-ranging seals are likely experiencing (Boily, 1996). Natural occurrences that happen to seals in the wild, such as molting and estrus, can be monitored in captive facilities (Ashwell-Erickson et al., 1985; Boily, 1996; Hall et al. 1998; Renouf and Noseworthy, 1990).

Food intake and morphometric measurements are important factors for captive animal management. Relations between mass and overall body length gain should be positively correlated to amount of food intake (Renouf and Noseworthy, 1990). Theoretically, the more energy in terms of food that an animal ingests, the larger or more mass the animal should gain. By examining the food intake and morphometrics of captive animals, models can be made seasonally, by sex differences, or by life history stages, such as molting or breeding (Renouf and Noseworthy, 1990; Boily, 1996). Energy intake is distributed differently in animals depending on where the metabolic demand is needed at the time of measurement (Renouf and Noseworthy, 1990; St. Aubin et al., 1996).

Sampling captive seals over time is an efficient way of establishing seasonal and time-related blood parameter trends and hormone levels. Using a relatively controlled research environment, monthly blood results and metabolic hormone samples, an outline of an animal's health can be mapped and potential health conditions, both endocrinological and immunological, can be evaluated. By using hormone ranges and morphometrics, captive facilities can examine each animal periodically to prevent diminishing health issues (St. Aubin et al., 1996). In addition, the comparison of captive animal health parameters with animals being admitted to rehabilitation can be diagnostic. This information can become the foundation for baseline health diagnosis data in captive and rehabilitation facilities.

Thyroxine (T4) is one of the thyroid hormones secreted via the thyroid glands, and is stimulated by thyroid stimulating hormone (TSH). Thyroxine aids in influencing aspects of reproduction, growth, metabolism and development of the immune system (Hall et al., 1998, Woldstad et al. 1999, St. Aubin and Geraci, 1987). During periods of distress, thyroid hormone concentrations decrease as a larger part of a feedback system to aid in conserving energy reserves (St. Aubin and Geraci, 1987; Oki and Atkinson, 2004). Most measurements of thyroid hormones, such as thyroxine, in all ages of an animal are remarkably constant. Findings from studies of beluga whales brought into captivity by St. Aubin and Geraci (1987), the glucocorticoid component of the stress response realigns to conserve thyroid hormone balance. Due to the negative feedback loop present in hormone relationships, decreases in circulating glucocorticoid levels elicit a compensatory increase in thyroid concentrations (St. Aubin and Geraci, 1987). Under stress, cortisol can increase

when capture and handling are involved. The opposite happens to thyroxine, there is a coincident decrease when capture and handling are involved. In thyroxine stress tests, thyroxine did not show a marked decline until 20 hours post sampling (St. Aubin and Geraci, 1987).

Cortisol is the dominant circulating glucocorticoid in marine mammals (Ortiz and Worthy, 2000). Cortisol is a steroid hormone, which is released directly from the adrenal cortex, stimulated by the anterior pituitary gland. Cortisol acts to maintain a level of homeostasis in the body to aid against environmental pressures (St. Aubin et al., 1996). Cortisol acts upon protein, lipid and carbohydrate mobilization and metabolism, aids in varied adaptations of the immune reaction to a number of distresses, such as allowing T-cells to self-regulate and producing several enzymes for pathogenic and anti-inflammatory response, thus limiting cell and tissue damage by doing so (Eckert et al.1988, Weber, 1997). Glucocorticoids, such as cortisol, mobilize immune function by declining their concentration in serum (Weber, 1997). Cortisol is an indicator of health status in an animal due to levels rising during times of stress (Oki and Atkinson, 2004). Cortisol is suggested to increase within 25 to 30 minutes after initial stressor (St. Aubin, 2001).

Molting in pacific harbor seals usually occurs in the late summer to early autumn months, June to October (Ashwell-Erickson et al., 1985; Riviere, 1978). Each harbor seals' molting period in captivity can differ; however an average time is approximately 5-7 weeks (Christen, ASLC unpublished data).

This study was conducted to continue to document captive harbor seal hormone ranges, following the circadian pattern study (Oki and Atkinson, 2004). The longer the consecutive time series of data on the same animals, the more useful the information becomes to describe patterns and anomalies. The captive harbor seal hormones were going to be used as a secondary comparison, as a group, to the rehabilitated harbor seals studied in the other part of this project. With the introduction of the wild harbor seal samples presented to the project, the captive harbor seal data has now become a small study of their own, or as a minor comparison group. These 22 consecutive months of data are important to document relationships between the metabolic hormones, TT4 and cortisol, feed intake and body condition.

Methods

Sample Acquisition

Captive harbor seals (n=7), three males and four females of varying ages, housed at the Alaska SeaLife Center in Seward, AK were sampled monthly. Some of the captive harbor seals bleeds were taken "voluntarily" with only behavioral cues. Some of the captive seals were physically restrained. Restraining methods included restraint boards with straps and head restraints with upright boards for blocking movement, hoop nets, restraint boxes, restraint stretchers and passive restraining. All behaviors with bleeds are described in the husbandry animal care charts of each animal. Blood was taken with an 18 gauge needle using extradural venipuncture. The volume of blood taken from each animal at each bleed was approximately 30 ml, but was determined by what diagnostics needed to be tested that particular month. Approximate maximum time of restraint was

noted to be 20 minutes. The average time of a successful first attempt in any of the restraint methods of was 5-7 minutes (Christen, personal communication). The serum was frozen at –70 degrees C until analysis. However, in some cases when serum was unavailable, plasma was validated and used *in lieu* of serum (Oki, 2001). One animal, Poco, was removed from the study since she died in the middle of the second sampling year of an esophageal carcinoma.

Radioimmunoassay Analysis

Hormone concentrations were measured using solid phase radioimmunoassay kits specific for cortisol or total thyroxine (TT4) (Diagnostic Products Corporation, Los Angeles, CA). Mean non-specific binding for TT4 and cortisol were 1.10% and 1.01%, respectively. Quality control indicators were included in each assay and provided by DPC. Pooled samples from young captive harbor seals were run in each assay as a method to determine and track interassay variation. Intraassay variation was less than 5% and interassay variation for all assays was less than 10%. Sensitivity of the assays for TT4 and cortisol were 0.33ng/tube and 4.5ng/tube, respectively. The standard curves of each assay were log-logit transformed, enabling extrapolation of sample concentrations (Rodbard, 1974). Parallelism was validated using a 25%, 50%, 100%, 200% doubling effect on the calibrator and pooled sample to determine the curve for both cortisol and TT4.

Morphometrics

Morphometrics were taken at each bleed for all animals. Morphometrics consisted of standard length (cm), weight (kg), axillary girth, hip and maximum girth

measurements (cm). These were made with a standard flexible tape measure and animals were weighed on a Cardinal Floor Hugger 4" x 4" platform scale, 708S digital rated for a maximum weight of 2000 pounds. Not all measurements were taken each time due to time constraints or the behavior of the animal. Only weight and length were used to make any correlations in this study due to inconsistent measurements with the other forms (girth, axillary and hip).

Diet Intake

Several diet studies were being performed during the 22 month sample period. Diet studies included such things as prey selection and daily intake of fish prey species. The diet intake that will be briefly discussed in this paper is focusing on gross daily intake of fish per animal (kcal). This is not further divided by prey type fed to the animals. Diet intake is used mainly as a corollary subject for mass gain.

Statistics

Mean and standard error hormone concentrations were calculated and categorized: 1) per hormone per animal, 2) per sampling year, and 3) per group of animals (divided by sex). One way ANOVAs were run on groups of seals to see if there was a difference in the means of hormone concentrations of the harbor seal pups, and in pre-wean and post-wean pups in rehabilitation compared to weaned pups in the wild population and the captive harbor seals adults. Graphs of all morphometrics or hormone concentrations were plotted over time throughout the two-year study period and yearly.

Molting

Harbor seals housed at ASLC were noted to start their annual molt in September. Most of the animals' molts lasted for approximately six weeks. However, two animals had individual molting patterns that differed from the other animals. Animal care staff noted pre-molt signs before actual fur shedding began, such as decreased appetite or more time spent hauled out. Distinctive behavioral signs have been noted during molting periods in some seals (i.e., lethargy, decreased activity), however these signs have not been noted to change normal daily training ability.

Results

Morphometrics and Diet Intake

Diet intake and morphometrics were not always positively correlated. According the figures 3-5 and 3-6, the majority of the seals (n=6) showed a negative relationship between food intake and weight gain. There was a food intake peak in the winter period (n=4) in 2001.There was also a slight food intake peak in mid-summer (n=4) in 2001. These were not the same animals in both peak periods. Males (n=2) showed an intake peak in late summer (July and August) and a decline in mid July (n=2) and the females peaked pattern (n=2, N=3) in December and intake declined in mid autumn (September to October). The females (n=2) seemed to have higher weight peaks in the mid winter (December to March). Males showed no pattern in body mass measurements by season except a decline in mass in the late summer months (August to September). Females showed a slight pattern (n=2) of mass declines in the late winter, early spring (April and May) that was not significant (Figure 3-1). Mass decline patterns do not change based on

sex, however with one animal, Skeezix, an adult female (Figure 3-5), there does seem to be a relationship between seasonality and mass. There was a pattern of massive food intake and then in the next month or two there was an increase in mass and then the food intake declined at that increasing mass. There was no consistent lag time between these two parameters. The patterns are playing a type of catch-up where mass gain declines while food intake decrease and vice versa so when graphed, the two lines form mirror lines, or opposites. This is illustrated in two example graphs using two of the male seals (Figures 3-5, 3-6).

Hormones-Thyroxine

The sample size of captive seals, in terms of comparison of individual animals and total thyroxine (TT4), per 22 month period is N=6. The majority of the animals during the November 2001 bleed (n=5), exhibited the lowest level of individual TT4 (mean=2.13 ng/ml, SE=0.16) for that sample year. A majority of the animals (n=5) had a TT4 peak during the period between the 2001 July and August bleeds (mean=4.68 ng/ml, SE=2.11). All of the animals (n=6) had a slight to significant peak during the September 2002 bleed (mean=3.57 ng/ml, SE= 0.55) (Figure 3-2). There was a slight pattern with 2 of each of the 3 animals in each sex category, meaning that n=2 for both males and females followed a seasonal pattern with TT4 for the 22 month sampling period; peaking slightly in late summer 2001 and early autumn in 2002 (both males and females) and had declines during late spring and mid-winter in 2001 (females). All males (n=3) had peak levels of TT4 in the warmer months (June to September) (mean=4.8 ng/ml, SE=0.27)

whereas the females (n=2) had significantly higher peaks in mid-winter (January and February), independent of years sampled (mean=4.9 ng/ml, SE=0.24).

Hormones-Cortisol

Cortisol in males seemed to peak in mid-winter (mean=16.22 ng/ml, SE=2.5) (January and February, n=2) and in females was elevated in mid- to late winter (mean=24.22 ng/ml, SE=1.4) (February and March, n=2). However the females had no pattern of declining cortisol ranges throughout the year. One female (Skeezix) had a peak in late August 2001. She exhibited the highest peak in cortisol of all the animals over the sampled 22 month period (<40ng/ml). Another female (Sydney) had peaks in the summers (June) and declined in the winters (January).

Figure 3-3 compiles all of the seven captive harbor seals sampled with their mean hormone concentrations for each monthly bleed. Figure 3-4 depicts means and standard errors of both hormones in all captive seals. Note that in Figure 3-4, from December of 2000 to January of 2002, the two hormones complement each other and actually show a similar pattern. In January of 2002, they began to have the opposite patterns; for example when cortisol began to peak in late January of 2002, TT4 declined. The 22 month period had differing patterns from year to year and it may be hypothesized that this is due to changing diet patterns, not determined by prey type, just referring to amount of intake. It may also be attributed to seasonal hormone fluctuations from events such as molting or estrus. However, the female captive seals were also on birth control.

Molting

The majority of the captive harbor seals exhibited hormone changes seasonally. No animal (N=6) showed a distinct change in hormone concentrations changing around the time of the annual molt. This might be due to the sampling frequency being insufficient to measure each animal molting at different times and at differing rates. Even though cortisol and thyroxine are noted in literature regularly as being hormones that are affected by the molt, these harbor seals did not show a distinct pattern of either of the hormones during the expected time of the molt as noted by the staff. The husbandry staff indicated that molting usually began in September and on average lasted approximately 6 weeks. The darker pelage seals show pre-molt signs well before an actual shedding began. One animal in particular (Pender) was noted to start later and molt longer than the other animals (Christen, personal communication).

Discussion

Total thyroxine has been noted to be negatively correlated to mass increase but positively correlated to food intake (Renouf and Noseworthy, 1990). However, in the same study, thyroxine levels were relatively low when mass accumulation and increased food intake were compared. Thyroxine levels were lower than baseline when correlated to metabolic rate. In other studies using captive seals, there was a negative relationship between mass gain and food intake (Boily, 1996). Some seals in captivity showed a decrease in food intake, which induced a depressed metabolic rate during the molt (Boily, 1996). When using food intake and mass accumulation in relation to each other, overall, thyroxine was at its lower limits (Renouf and Noseworthy, 1990). Mass gain and intake

were negatively correlated to each other in the majority of the seals. Comparing Figure 3-1 and Figure 3-2, there is a negative relationship between TT4 increases and mass gain. There was no detectable pattern between TT4 and mass gain.

In St.Aubin et al. (1996), sex was determined as one of the most influential factors on thyroxine concentrations in *Tursiops truncatus* with higher concentrations found in females. There were some differences in total thyroxine levels between the male and female harbor seals during the 22 month study, even though they are different species. These differences were not significant ($p>0.05$). There were some differences in the present study, however, individual animals' TT4 fluctuated more often than TT4 between sexes. Thyroxine has been documented to decline in lower seasonal temperatures to conserve energy (Bubenik and Brown, 1989; St. Aubin et al., 1996).

Cortisol did not show large depressions or peaks across the 22 month study period. The peaks and depressions also did not correlate to food intake. Cortisol changes metabolic activity and there would be an assumption that is would be altered by food intake and weight adjustments. Animals in captivity do not always mirror natural occurrences normally found in the environment in the same timeframe as they would in the wild. Sometimes molt periods are shorter or begin weeks later when comparing the wild animals to the animals in captivity. Without using hormones that are critical in the molting process (i.e., cortisol, thyroxine, and melatonin) (Riviere et al., 1977; Ashwell-Erickson et al., 1986), or certain blood parameters to determine when the actual molting period of individual animals actually begins, no accurate comparison of seals in captivity to seals in the wild is possible. High levels of cortisol have been noted in molting seals,

generally with an inverse relationship to thyroid hormones (Riviere et al., 1977; Ashwell-Erickson et al., 1986). This would make sense going back to the feedback compensatory loop that cortisol and thyroxine possess; that when one hormone increases, the other decreases to conserve energy.

Alaskan harbor and spotted seals were recorded to have an average molt time that ranged form 120 to 170 days from first molt sign to full fur emergence on the back (Ashwell-Erickson et al., 1985). ASLC staff recorded an average of 6 weeks for the seals in the facility to molt and this is normally noted in September (ASLC husbandry notes, unpublished). Boily (1996) states that seals in captivity showed a decrease in food intake and lowered metabolism with lower total thyroxine levels during the summer molt. Ashwell-Erickson et al., (1985), state that cortisol levels were at their maxima just prior to major shedding events during the molt period and at their lowest levels during the new hair growth during the molt period. In the same study, total thyroxine levels were lower early in the molt and at their maximum toward the end of the rapid new hair growth period. Thyroxine was not measured during the new hair growth period of the molt (Boily, 1996). Using this information, total thyroxine and cortisol counter each other's activity during the molting period (Riviere et al., 1977). Ashwell-Erickson et al., (1985), stated that cortisol might mobilize fat reserves during the molt period, leading to mass reduction during and directly following the major molt period. Determining the actual onset of the molt is complicated, due to the fluctuation of the thyroid hormones during the unobserved portion of the molt: the follicular stimulation (Renouf and Brotea, 1991; St. Aubin, 2001) which is when the thyroid hormones increase. The sampling period of

molt in captive seals should begin approximately one to two weeks before the actual observed onset of molt, noted by decreased appetite, behavioral changes and actual hair shedding. In summary, this shows that using harbor seals in a captive situation as control specimens can aid in relationships between hormone changes and stress in seals not accustomed to daily handling.

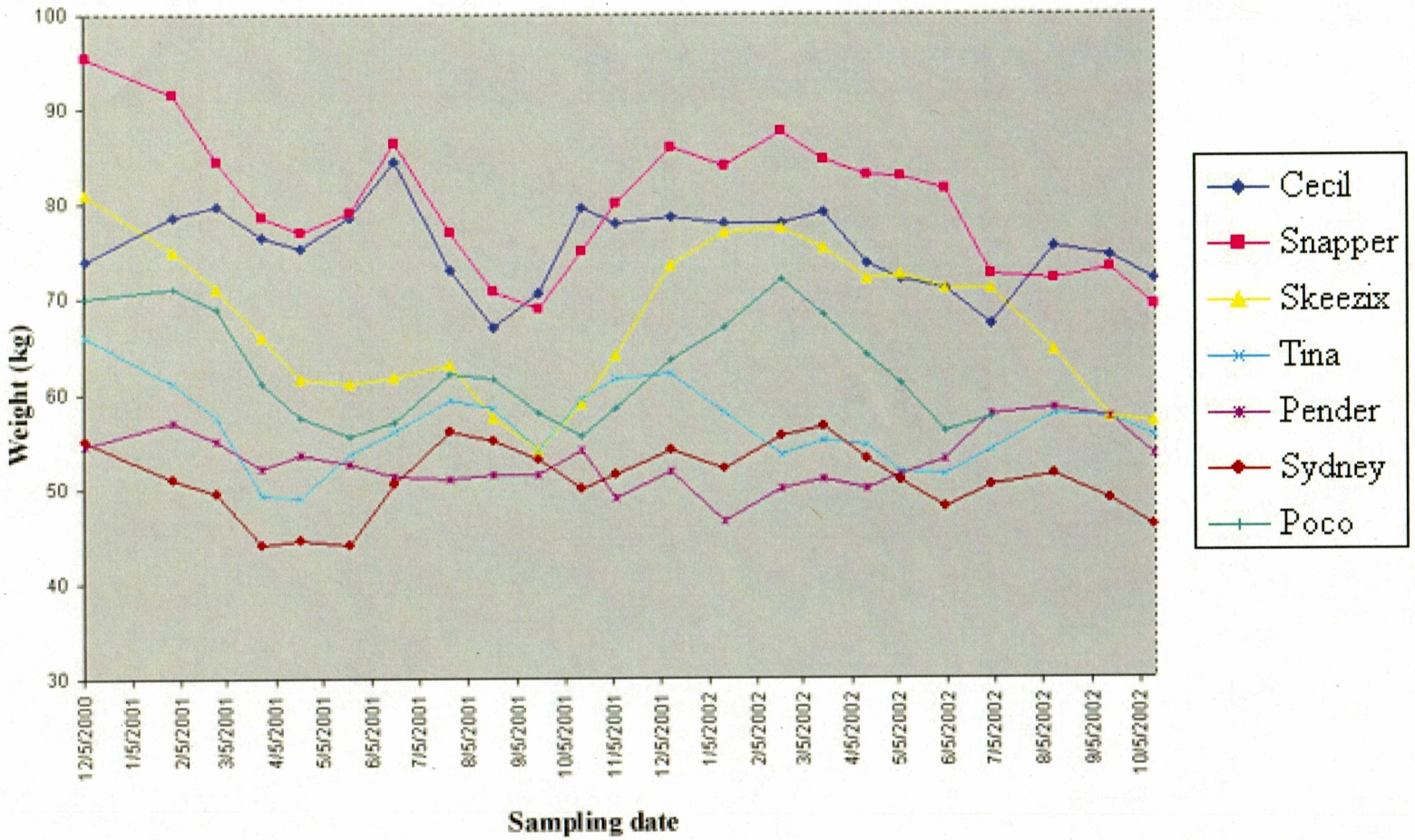

Figure 3-1: Captive Harbor Seals' Weight (kg) Across 22 Months Plotted by Date

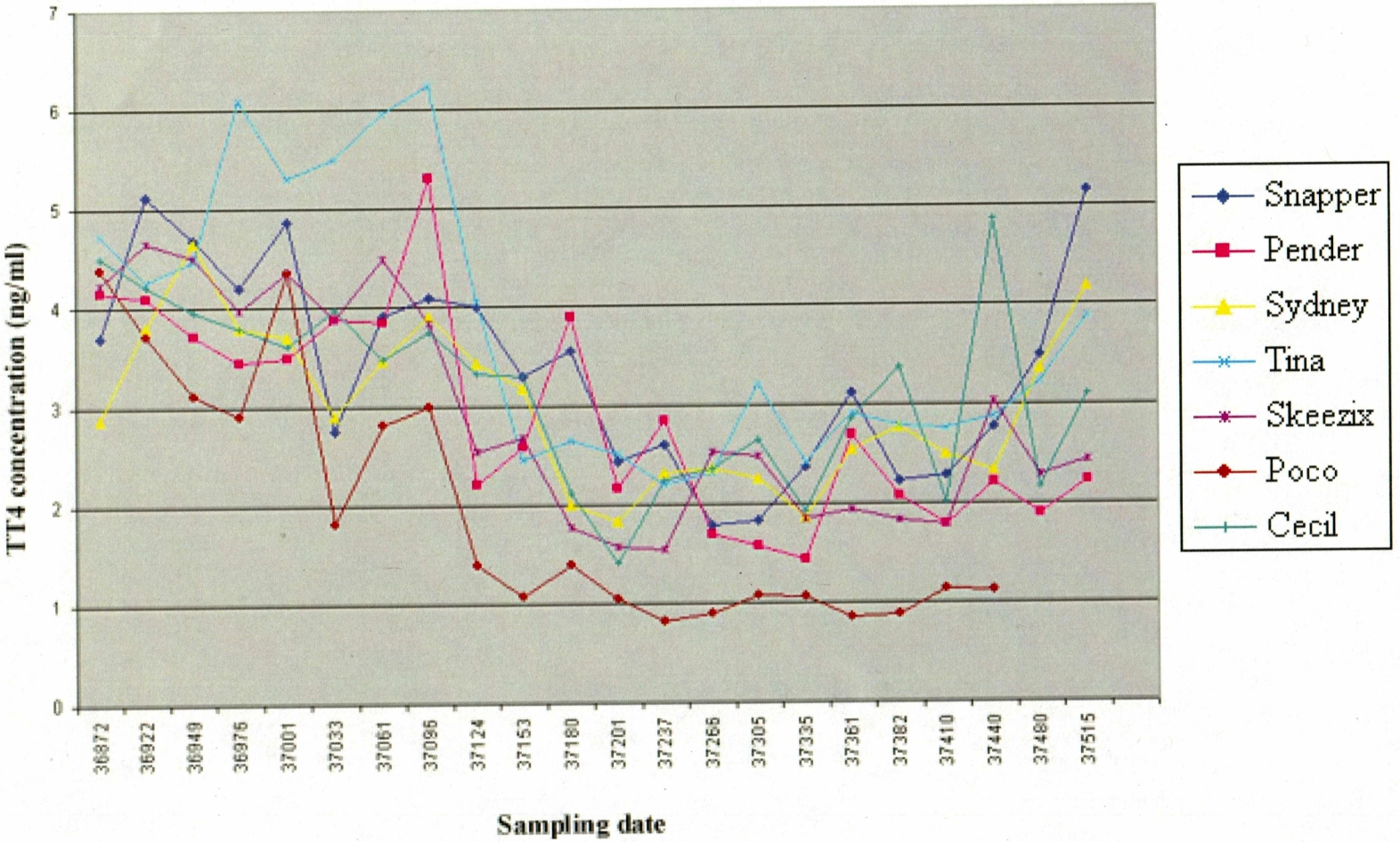

Figure 3-2: Captive Harbor Seals' TT4 (Total Thyroxine) Profiles for 22 Months Plotted by Date

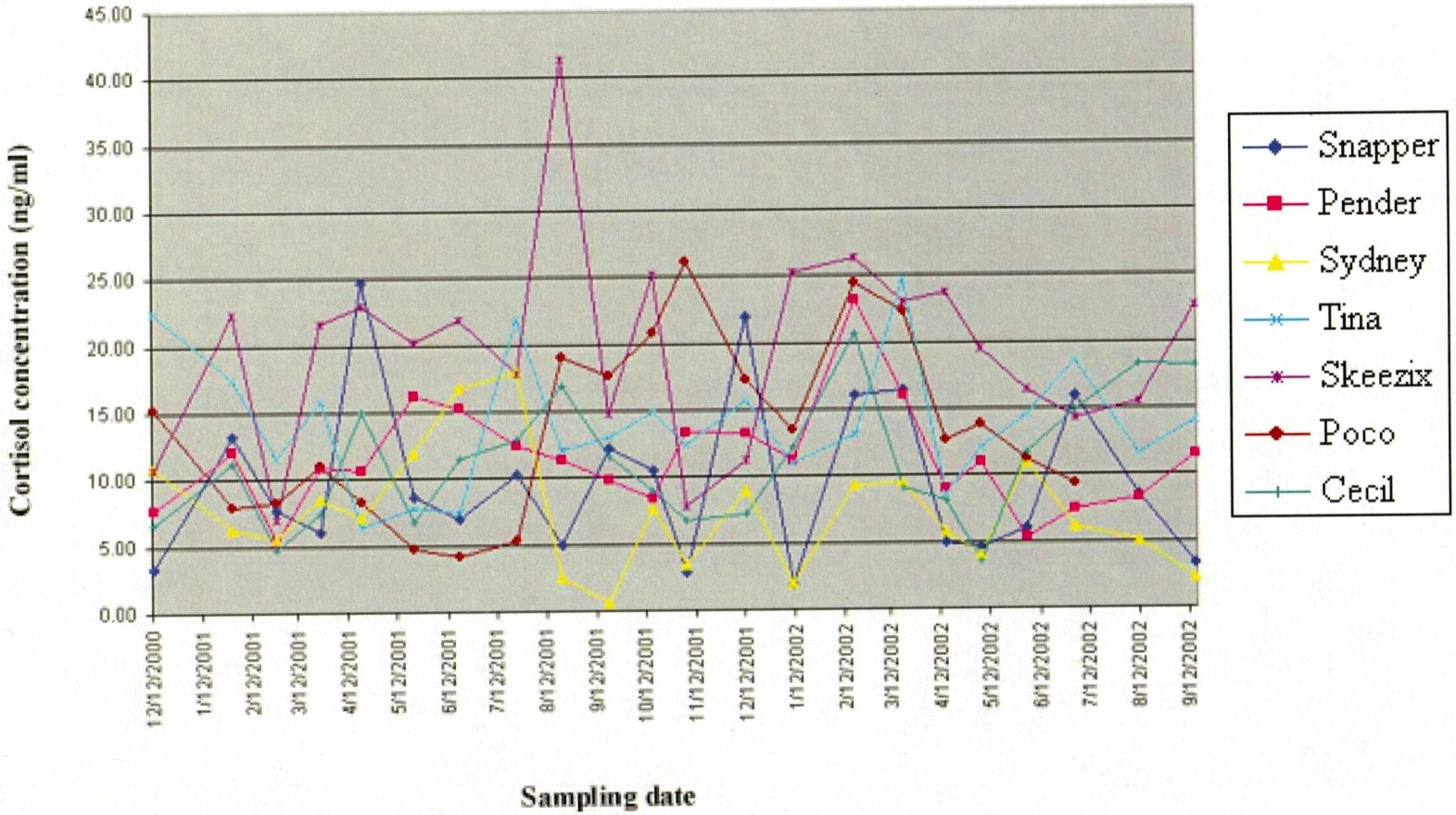

Figure 3-3: Captive Harbor Seals' Cortisol Profiles for 22 Months Plotted by Date

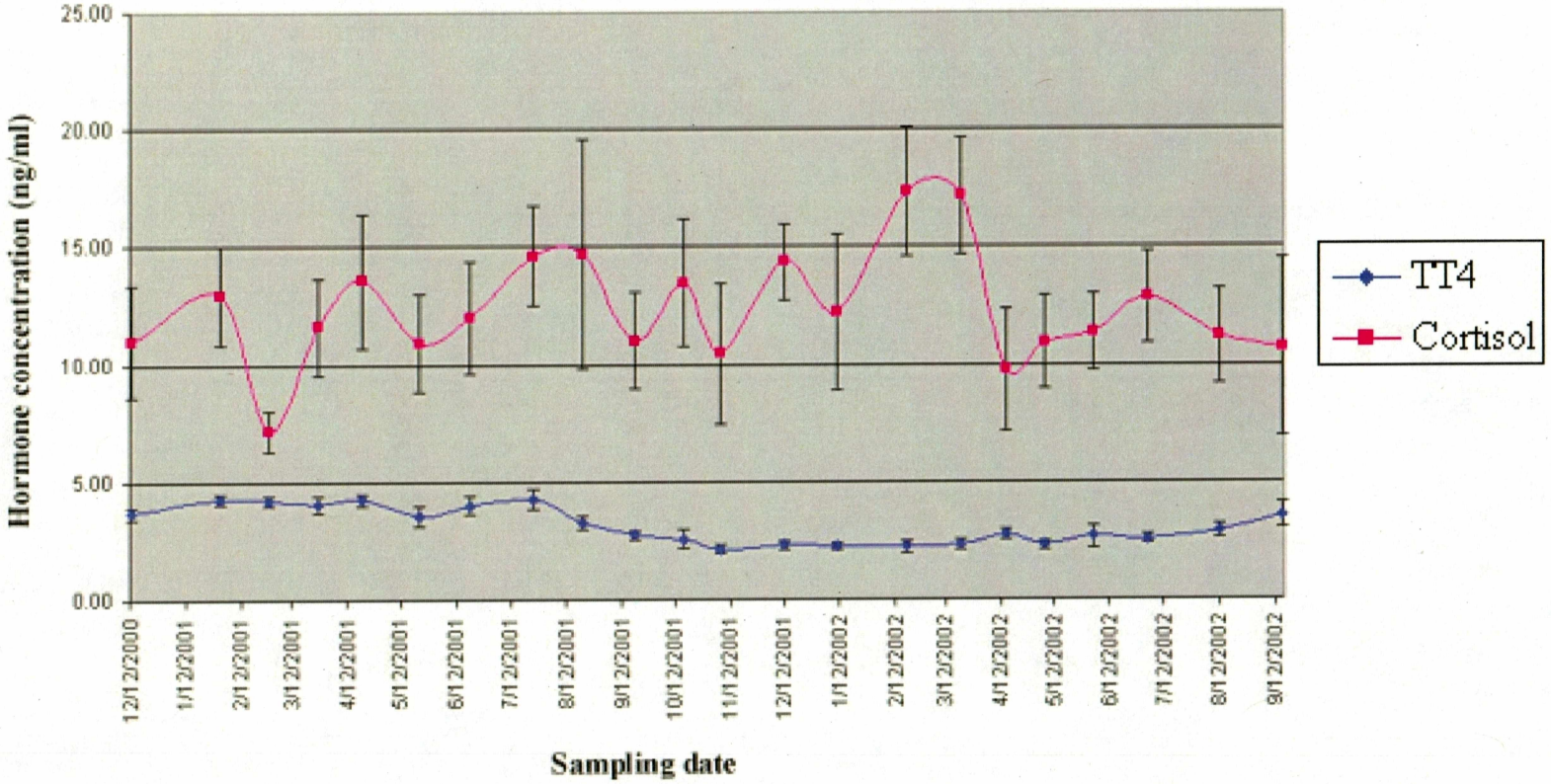

Figure 3-4: Captive Harbor Seal Hormone Ranges over 22 Months by Bleed Date with Standard Error

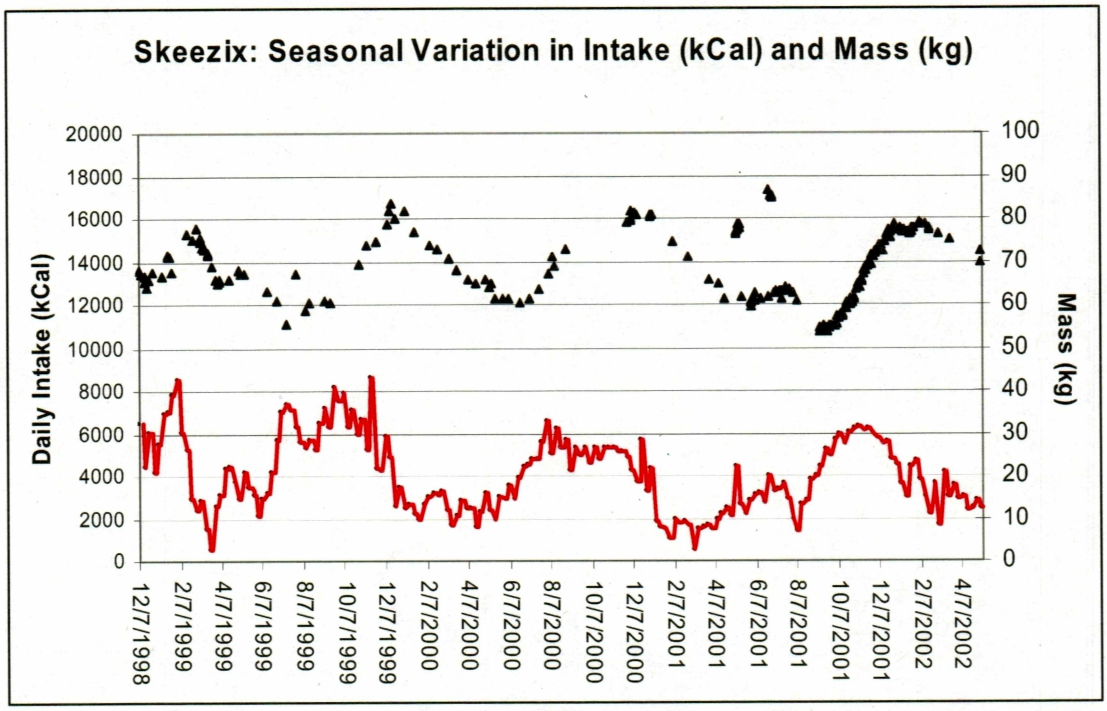

Figure 3-5: Captive Harbor Seal, Skeezix, Seasonal Variation in Food Intake (kcal) and Mass (kg)

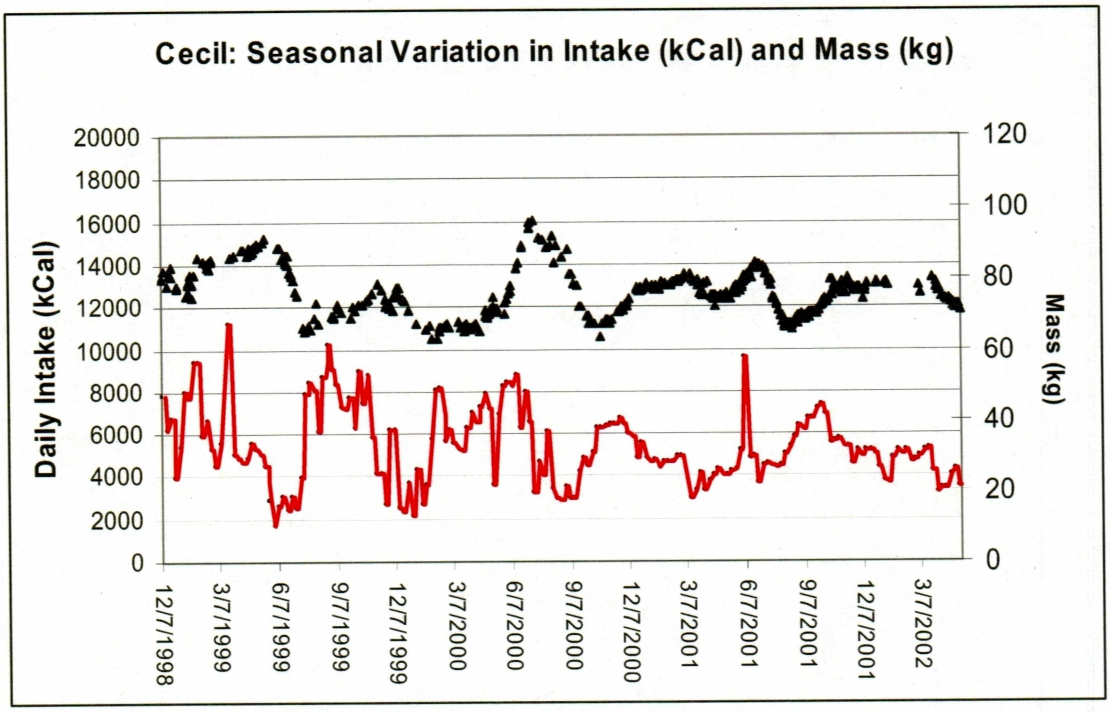

Figure 3-6: Captive Harbor Seal, Cecil, Seasonal Variation in Food Intake (kcal) and Mass (kg)

Chapter 4 The Big Picture

Wild Harbor Seal Pups Compared to Rehabilitated Harbor Seal Pups

As demonstrated in Chapter 2, the wild harbor seal pups sampled had the highest mean concentration of total thyroxine (TT4) sampled. The wild pups also exhibited the largest counts of lymphocytes. Morphologically, they were much larger in mass and length than the rehabilitation pups. Pups sampled in the wild were considered for the purpose of this study to be a representative of the free-ranging population, at least in the area sampled (Tugidak Island, Alaska). According to natural selection for phocids, the larger the animal with a higher amount of fat at birth, the better the fitness of the animal. If this is applied to the wild pups in this study, then the larger mass and length trend seen with the wild pups shows that they are healthier and have a greater likelihood for survival to reproductive maturity. However, the rehabilitation pups were not a uniform population as they came from different areas in Alaska and California. Alaska harbor seal pups are larger compared to the California harbor seal pups, most likely due to these animals needing to be a larger birth mass to combat colder climates and water temperatures at birth (Wynne, 1997). The animals undergoing rehabilitation were compromised in some way, exhibiting disease processes, or the victims of maternal abandonment, attempted predation or human interaction. A large percentage of pups were admitted to rehabilitation as neonates (younger than one week) making comparison with the wild pups problematical, since theoretically, all of the wild pups sampled were close to weaning age. With this taken into account, the real comparison must be between post-weaned pups and the wild pups. This lowers the sample size of the rehabilitation group.

Taking this into consideration, the rehabilitation pups were presented as a test group that may serve as bioindicators for a species that is still considered not recovered in the Gulf of Alaska after EVOS (Exxon Valdez Oil Spill Trustees Council). Pups with fatal or long-term disease processes were divided out of the group, prior to any statistical process, based mainly on lack of serial serum samples needed to establish a proper baseline (minimum of three serial samples), per individual animal. The sample size was larger for wild pups, however, there was not a significant difference statistically.

The wild pups showed higher TT4 concentrations, larger size and higher percentage of lymphocytes compared to both pre-weaned and post-weaned rehabilitated pups. According to the research team that sampled these wild pups, the animals were not chased, harassed or held longer than 20 minutes prior to bleeding. Thus, the thyroid hormone concentrations shown, as stated by the literature, are likely to be resting values and not stress-induced artifacts. The higher comparable hormone concentrations may mean that wild pups are dealing successfully with elevated acute stressors in the environment such as; thermoregulation, foraging energetics and predatory threats. Some of these wild pups might also be dealing with chronic stressors that have affected their immune systems, hence, the higher percentages of lymphocytes (Figure 2-3), or possibly their immune systems are more mature.

The most complicated aspect of studying a stress response in an animal is the difficulty in determining the difference between the true stress state versus an unstressed, or baseline state. Stressors that the rehabilitation pups might have been dealing with while in rehabilitation were events such as emaciation, disease, the introduction of

another pool mate, live fish or a pool or pen move. Stressors such as thermoregulation or predation were either reduced or eliminated. However, sometimes events, such as temperature fluctuation and competition for prey, that seal pups would normally be dealing with in the wild were left to affect the individual animal, if the animal were close to release, out of quarantine, off of any medications and deemed healthy, meaning no detectable disease state or ailment. Overall, animals in rehabilitation, especially while in critical care, were kept away from stressors as much as possible. Human interaction is always an issue to an animal's health and complicated to erase from rehabilitation, especially when animals are neonatal. Good rehabilitation practice does attempt to minimize contact with animals. Training with staff and volunteers seeks to minimize stress to an animal via noise, handling and over sensory activity (people watching). This is why research like this project, using stress hormones in a species that strands often, is necessary to hone skills for marine mammal rehabilitation centers.

Captive Seals

Captive seals from the Alaska SeaLife Center were not used as a comparison group to the rehabilitation or wild population seals for three main reasons. The first reason is that the ages of the animals are very different. With different ages, come ontogenetic differences in the immune and endocrine systems as well as metabolic demands. Older seals molt annually. This molt was associated with varying metabolic hormone levels, including cortisol and total thyroxine (TT4), even though as seen in Chapter 3, the captive seals did not show a strong correlation between either of the metabolic hormones, and molt. The molting period encompasses enough changes

involving the two hormones studied that a valid comparison was not warranted with the pups. The second reason is due to the adult seals exhibiting seasonal metabolic changes. Each sex has showed different seasonal patterns in terms of the two hormones studied, it, too, is not a stable comparison for the pups. The third reason is that the animals are captive animals. These animals have become habituated to human interaction in a captive setting and probably showed dampened stress responses both immunologically or hormonally to stressors, (predation, temperature, foraging) and thus are an inappropriate comparison group for the pups.

Hormone research with marine mammals is still an evolving topic. Though numerous researchers have helped to pioneer the field of endocrinology and immunology in marine mammals, the future might include using rehabilitation centers for the basis of new studies, instead of using large numbers of wild animals. Though the complexity of trying to control for factors such as age, disease and stranding stress in animals admitted to rehabilitation centers is complicated, the usefulness of animals that present in large sample sizes is very helpful to researchers. Realizing that human interaction cannot be completely excluded from baseline data, it can be controlled for, such as time limits in handling the animals for studies including hormone action and stress related physiology.

The big picture of this study was to show the usefulness of biomarkers to indicate endocrine and immune function in animals from rehabilitation facilities and for the care and release of viable animals back into the wild population, by utilizing healthy rehabilitation practices. This was demonstrated using hormone and immune studies with harbor seal pups and comparing these results with wild pups. Overall most rehabilitated

pups were able to acclimate to the rehabilitation environment and deal with the rehabilitation procedures, as evidenced by the decrease in cortisol concentration during the rehabilitation process from pre-weaned to post-weaned. It suggests that the rehabilitation facilities were following procedures that do not overtly disturb an animal that may have recovered from a disease process or an injury, and were releasing immunologically and endocrinologically comparable animals back into the wild populations. However, future research is necessary to continue to use the technology available in the scientific community to monitor and treat animals coming into rehabilitation, no matter why these animals are being admitted, whether it is human interaction (handling, entanglement, gunshots), pollution (food web deficiencies or toxins such as organocholorines), other disease processes in the marine environment, or natural perturbations in the environment that are somehow impairing harbor seal populations.

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